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Science and Advice for Scottish Agriculture

THE BIODIVERSITY AND EPIDEMIOLOGY OF *POTATO VIRUS Y* (PVY) IN SCOTLAND

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Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

DECEMBER 2014

Abstract

Potato virus Y (PVY) is considered to be the most serious viral pathogen that affects potato crops worldwide and can cause substantial yield losses. PVY exists as a complex of strains that can be distinguished on the basis of their biology, serology and genome analysis. In recent decades novel recombinant PVY^{N} strains have emerged that can cause Potato Tuber Necrotic Ringspot Disease (PTNRD). It is therefore important to understand the potential threat to the Scottish seed potato industry. This molecular nature of PVY isolates in Scotland was established through the use of partial sequencing, revealing a predominance of isolates belonging to the molecular EU-NTN clade (ca 75%). Assessing the biological characteristics of selected isolates indicated that most isolates in Scotland belong to the biological PVY^{N} type, however PVY^{E} is also present. Molecular analysis of a PVY^{E} isolate has shown that identifying the molecular determinants for vein necrosis production in tobacco is complex. Although it has not been reported from the field in Scotland, PTNRD initiation is possible with most PVY^{N} isolates under optimal climatic conditions. Field trials suggest that $PVY^{EU\text{-}NTN}$ is more efficiently transmitted by aphids across a growing season than $PVY^{NA\text{-}NTN}$ and PVY^{O}, with a higher than expected proportion of tubers infected with the $PVY^{EU\text{-}NTN}$ isolate. This suggests that once plants are inoculated with the virus, $PVY^{EU\text{-}NTN}$ isolates are more likely to infect progeny tubers. Taken together, the outcomes of this project should provide a better understanding as of PVY molecular nature in Scotland its pathogenicity and epidemiology with the view to understanding why PVY^{N} variants have become an important threat for the seed potato industry both in Scotland and worldwide.

Publications arising from this work

Davie K, Lacomme D & Dickinson M (2012) The biodiversity and epidemiology of *Potato virus Y* in Scotland, Proceedings Crop Protection in Northern Britain.

Lacomme C, Davie K, Dickinson M (2013). The biodiversity and epidemiology of *Potato virus Y* in Scotland. *Potato Research* special edition abstracts European Association Potato Research Virology.

Lacomme C, Davie K, Holmes R & Dickinson M (2014). PVY^{N} prevalence in potato crops: impact of strain competition and ability to overcome plant resistance mechanisms. Proceedings Crop Protection in Northern Britain.

Acknowledgements

It is a great honour and privilege to be able to thank the following people for their help and support throughout this project. Firstly I would like to thank my supervisor **Dr. Christophe Lacomme** who was always available for advice and support throughout the project. He has provided me with constant encouragement and enthusiasm for this project. I must also thank **Professor Matthew Dickinson** for his advice and support in relation to this project. Various staff at SASA have helped with this project. A heart felt thank you must go to all of the Virology team who assisted when they could in respect to helping me where getting through the work was a difficult task **Ross Holmes, Katy Thomson,** and **Barry Hall** all deserve a special mention. Thanks to the photography department for taking images as requested. I also must thank the horticultural staff at SASA for providing me with fresh plants on a weekly basis and helping me with the summer field trial. **Sandra Goodfellow** is thanked for providing micropropogated plants for my trials. Horticulture and farm staff were invaluable in planting and harvesting of trials. The staff in diagnostics and molecular biology were always happy to help and a special mention should go to **Vince Mulholland** who talked me through the complexities sequence analysis. On a personal level I would like to thank all of the Zoology team at SASA, especially the "girlies" and also my flatmate **Kara Dicks**, as they have been a constant source of support and friendship throughout this project. Finally, I would like to thank the British Potato Council for providing funding towards this work.

Abbreviations

BPC	British Potato Council
BYDV	Barley Yellow Dwarf Virus
CI	Cytoplasmic Inclusion
CMV	Cucumber Mosaic Virus
CP	Capsid/Coat Protein
CV	Cultivar
DAS-ELISA	Double Antibody Sandwich - Enzyme Linked Immunosorbent Assay
DI	Disease Index
cDNA	Complimentary Deoxyribonucleic Acid
DPI	Days Post Infection
DV	Diseased Variety
ER	Extreme Resistance
EU-NTN	European PVY^N Tuber Necrosis
FERA	The Food and Environment Research Agency
HC-Pro	Helper Component Proteinase
HR	Hypersensitive Response
MEGA	Molecular Evolutionary Genetics Analysis
N	Necrosis (where PVY^N this relates to vein necrosis in tobacco)
NA	North American
NIa	Nuclear Inclusion Protein a
Nib	Nuclear Inclusion Protein b

NT	Nucleotide
N-Wilga	A recombinant strain of PVY that causes vein necrosis in tobacco
ORF	Open Reading Frame
P1	Proteinase 1
P3	Proteinase 3
PBST	Phosphate Buffered Saline
PIPO	Pretty Impressive Potyviridae ORF
PLRV	Potato Leafroll Virus
PTNRD	Potato Tuber Necrotic Ringspot Disease
PVA	Potato virus A
PVX	Potato virus X
RdRp	RNA-dependent RNA polymerase
REF	Relative Efficiency Factor
RNA	Ribonucleic Acid
mRNA	Messenger Ribonucleic Acid
ssRNA	Single Stranded Ribonucleic Acid
RJ	Recombinant Junction
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SASA	Science and Advice for Scottish Agriculture
SPCS	Seed Potato Classification Scheme
SNP	Single Nucleotide Polymorphism
S	Susceptible
TPS	True Potato Seed
UK	United Kingdom

VN	Vein Necrosis
VPg	Viral Genome-Linked Protein
VRC	Viral Replication Complex
6K1	6-kiloDalton protein 1
6K2	6-kiloDalton protein 2

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Chapter 1 - General Introduction

1.1. The seed potato industry: UK and worldwide

The cultivated potato, *Solanum tuberosum* ssp. *tuberosum*, is a member of the *Solanaceae* family which comprises of over 300 species. It belongs to the genus *Solanum* which contains over a thousand species including tomato, pepper and aubergine. The *Solanum tuberosum* species has two subspecies, these are *tuberosum* spp. and *andigena* spp. *Solanum tuberosum* originates from South America which is where the highest number of wild potato varieties can be found to this day. There are records dating back 8,000 years from Bolivia and Peru of potatoes being grown for human consumption (Spooner *et al*., 2005). The potato was first introduced into Europe through the Spanish conquests of South America during the 16th century and was widely cultivated throughout Europe by the middle of the 19th century. It can now be found in over 130 counties around the globe under different climatic conditions and is currently the 4th most important food crop in the world after maize, wheat and rice (Hawkes, 1990). Annually 373 million tons are produced worldwide and production is worth an estimated $6 billion dollars. China produces the greatest percentage of the global potato crop 24% (Singh *et al*., 2013), but North America has the highest yields in the world. The UK is the 12th largest potato growing country and one of the highest consumers at 90kg per person per annum. The British potato industry contributes approximately £731 million to the economy at leaving the farm gate and up to £5.7 billion at the consumer level (BPC, Market intelligence report 2012-13). It is the second most economically important crop grown in the UK after

wheat. The current land use area for potato production in the UK is 126,450 hectares, (Davies, 2010) which is far lower than the 250,000 hectare area grown in the 1960s (M^{c}Gregor, 2007). However, with the advances in agricultural technologies and disease prevention the yield per hectare has increased from 23 tonnes in the 1960s (M^{c}Gregor, 2007) to approximately 45 tonnes in 2010, making the overall yields similar over the past 50 years.

1.2 Potato production and classification in Scotland

Within Scotland alone the potato industry is thought to be worth an estimated £160M to the economy prior to processing. For the purposes of trade there are two categories of potato, those grown for consumption, known as 'ware' crops and 'seed' potatoes which are grown to produce more potatoes. As seed potatoes will be bulked up by several field generations it is vital that they are as healthy as possible. As a consequence there are limitations as to where seed crops can be grown (Van Loon, 2007).

The total area in Scotland used for potato production in 2013 was 29,000 Ha and this can be broken in to 16,500 ha and 12,600 ha of Seed. Scotland is recognised as an EU community grade region producing only basic and pre-basic seed (www.scotland.gov.uk/Topics/Statistics/Browse/Agriculture-Fisheries/agritopics/Pots). This community grade status requires uniform certification of regions involved and only seed grown in community grade regions can be introduced. Approximately 75% of the UK seed production comes from Scotland; the main reason for this is due to Scotland's cold wet and windy climate, which is unfavourable to aphid survival and incidence

resulting in a low level of virus. Seed potatoes in Scotland are regulated by The Scottish Seed Potatoes (Scotland) regulations 2000. All stages of production are under the strict control of the Scottish Government. SASA (Science and Advice for Scottish Agriculture) operates the Scottish Seed Potato Classification Scheme. Classification and marketing of seed potatoes comes under the Seed Potatoes (Scotland) regulations 2000 and its subsequent amendments.

In Scotland all seed potatoes originate from *in vitro* micropropagated tissue culture grown at SASA. The microplants are tested for a wide variety of diseases and off types and only the purest seedling are allowed to enter production. Nuclear stock microplants are distributed to a few approved growers for the purposes of multiplication of mini tubers. The multiplication process for seed potatoes in Scotland is usually 3 to 6 generations, more generational multiplications result a higher number of tubers produced but concomitantly with risk of diseases. The Scottish Seed Potato Classification Scheme (SPCS) aims to ensure that Scottish seed potatoes are high in health and purity. Only potato stocks which meet high standards in both crop and tuber inspections are allowed to be marketed as Scottish seed.

Inspections are carried out on all seed crops biannually to check for the presence of viral, bacterial and fungal pathogens and varietal impurities to ensure these levels are being met. Growing crops are monitored throughout the growing season and are officially inspected at least twice for virus diseases. Inspectors are trained to visually identify the 30 most commonly grown varieties in Scotland and to recognise bacterial and fungal disease symptoms

along with mild and severe mosaic which are caused by virus infection. There are different tolerances for pests and diseases for different seed potato grades which for viruses are based on the number of plants affected by either mild or severe mosaic symptoms within the crop. The purpose of seed certification is to provide assurances that transmissible pathogens are at an acceptably low level (Albrechtsen, 2006)

1.3 Potato-infecting viruses

Potatoes are susceptible to a wide array of pathogenic organisms including bacteria, fungi, nematodes, viruses and viroids. The effect on the crop can be losses in yield, tuber quality which result in poor marketability of tubers. Potato viruses are a major concern to the UK potato industry with estimated associated losses of £12M to £22M per annum (Twining *et al.*, 2009). At present there are over 40 known viruses that affect potato (Table 1.1, Valkonen, 2007). For many potato viruses once a tuber has become infected, any progeny generations from that tuber will also be infected and will provide the main source of inoculum known as seed transmission. The other method for transmission is by a vector. Potato viruses are transmitted in nature by a large variety of microorganisms, fungi, protist and invertebrates vectors including nematodes and several Hemipteran insect species including, most importantly, aphids. Of the viruses that infect potato, Polerovirus, (Potato leafroll virus, PLRV) Potyviruses (Potato virus Y –PVY - Potato virus A - PVA, Potato virus V -PVV), Carlaviruses (Potato virus M – PVM- and Potato virus S –PVS) and Potexvirus (Potato virus X - PVX) are found worldwide in potato growing areas (Valkonen, 2007).

Table 1.1 Viruses known to infect potato, adapted from Valkonen (2007)

Species	Occurance in Potato	Transmission by
Alfalfa mosaic virus	Worldwide	Aphids
Andean potato latent virus	South America	Beetles, TPS
Andean potato mottle virus	South America	Contact
Arracacha virus	Peru, Bolivia	TPS
Beet curly top virus	Worldwide (arid countries)	Leafhoppers
Cucumber mosaic virus	Worldwide	Contact, Aphids
Potato black ringspot virus	Peru	Nematodes?
Potato deforming mosaic virus	Brazil	Whiteflies
Potato latent virus	North America	Aphids
Potato leafroll virus	Worldwide	Aphids
Potato mop top virus	Peru, Europe	Spongospora subterranea
Potato rough dwarf virus	Agentina, Uruguay	Aphids
Potato virus A	Worldwide	Aphids
Potato virus M	Worldwide	Aphids
Potato virus S	Worldwide	Aphids
Potato virus T	South America	Contact, TPS, Pollen
Potato virus U	Peru	Nematodes?
Potato virus V	Europe, South America	Aphids
Potato virus X	Worldwide	Contact
Potato virus Y	Worldwide	Aphids
Potato yellow dwarf virus	North America	Leafhoppers
Potato yellow mosaic virus	Carribean	Whiteflies
Potato yellow vein virus	South America	Whiteflies
Potato yellowing virus	South America	Aphids, TPS
Solanum apical leaf curl virus	Peru	?
Sowbane mosaic virus	Worldwide	?
Tobacco mosaic virus	Worldwide	Contact
Tobacco necrosis virus	Europe, North America, Tunisia	Olpidium brassicae
Tobacco rattle virus	Worldwide	Nematodes
Tobacco ringspot virus	South America	TPS, Nematodes?
Tobacco streak virus	South America	?
Tobacco black ring virus	Europe	TPS, Nematodes
Tomato mosaic virus	Hungary	Contact
Tomato mottle taino virus	Cuba	Whiteflies
Tomato spotted wilt virus	Worldwide (hot countries)	Thrips

A ? indicates that the vector is unknown.

1.4 Population dynamics of potato-infecting viruses in the Scottish seed certification scheme

The data presented in Table 1.2 illustrate the relative proportion of cases (the number of crops in which at least one leaf sample is tested positive for a virus) of viruses found in symptomatic leaf samples during inspection of seed potatoes over the past seven years (SASA Virology Growing Crop report, 2012). Leaf samples taken from plants exhibiting disease symptoms (mosaic) are sent to SASA for ELISA testing. As presented in Table 1.2 the large majority of viruses belong to the potyvirus group (63% PVY^{N}, $PVY^{O/C}$, PVA, PVV). PVY^{N} (veinal necrotic type strain) is the most prevalent virus strain over the $PVY^{O/C}$ (Ordinary and C strain) and also all other virus species. From 2003 to 2010 there was a ten-fold increase in the number of cases of PVY^{N} intercepted in the field. PVY^{N} now accounts for approximately 50% of all viruses found (Figure 1.1).

Table 1.2 Number of virus cases found in submitted symptomatic potato leaf samples 2006-2012. (Data from SASA SPCS Growing Crop report 2012).

Virus	2006	2007	2008	2009	2010	2011	2012
Potato Leaf Roll Virus	51	48	107	160	57	20	21
Potato Virus YO/C	49	52	32	35	61	29	19
Potato Virus Y^{N} (VN)	117	153	184	299	376	174	153
Potato Virus A	56	103	108	75	78	58	33
Potato Virus V	11	32	30	13	23	12	3
Potato Virus M	0	0	0	0	0	0	0
Potato Virus S	0	2	2	1	0	1	8
Potato Virus X	23	38	18	19	20	25	29
Tomato Black Ring Virus	1	7	1	2	0	1	0
Tobacco Rattle Virus	32	53	19	3	9	1	10
Potato Mop Top Virus	10	52	40	17	23	49	54
Total	**350**	**540**	**541**	**624**	**647**	**370**	**330**

PVY in Scotland has been consistently the most commonly intercepted virus in Scottish Seed potatoes and in particular PVY^N. Surveys of virus population of seed potato crops in Scotland have identified a recent drift in the PVY population structure from PVY^O and PVY^C towards PVY^N (Figure 1.1) a trend observed in continental Europe (R. van der Vlugt, personal communication) suggesting the selection of potentially fitter PVY strains.

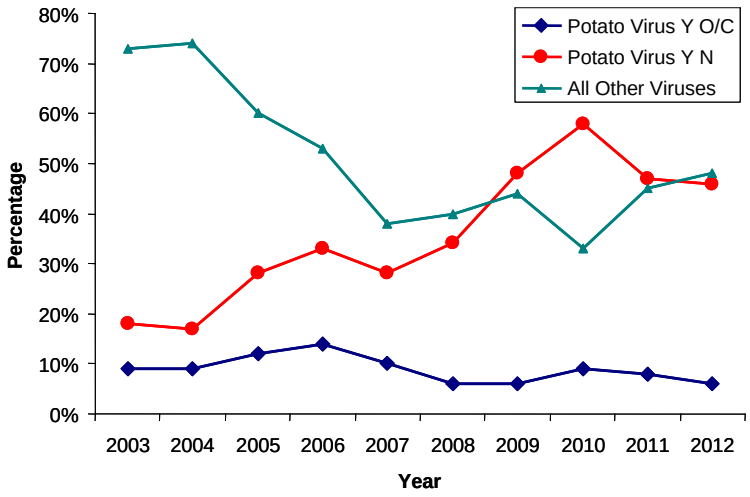


Figure 1.1 The percentage of $PVY^{O/C}$ or PVY^N cases identified by DAS-ELISA. The cumulative proportion of other diagnosed viruses described in Table 1.1 is presented (data from SASA SPCS Growing Crop report 2012).

1.5 Potyviruses and *Potato virus Y* (PVY).

The plant virus family *Potyviridae* is the largest plant virus family (Reichmann *et al*., 1992). It contains six genera and around 200 virus species, most of which have a monopartite positive single stranded (+) ssRNA genome. The largest genus in the *Potyviridae* family is the genus *Potyvirus* which contains 128 approved and 89 tentative species. All potyviruses are transmitted by aphids. Traditionally virus species and virus strains were defined on the basis of symptomatology, host plant range and serology. Sequencing techniques have enabled molecular taxonomy in closely related species. Using the amino acid sequences of the CP of virus species, the genus Potyvirus exhibit 38-71% similarity, while strains share 90-99% similarity (Adams *et al*., 2004). Potato virus Y (PVY) is the type member of the genus potyvirus (Hull, 2002) and has a worldwide distribution with a large host range which includes not only cultivated solanaceous species (potato, tobacco, tomato, pepper, petunia) but also many solanaceous and non-solanaceous weeds. PVY have single flexuous rod shaped particle of a modal length of 740nm consisting of over 2000 copies of a coat protein (CP) monomers with a genomic RNA polyadenylated tail at the 3'-end and a VPg at the 5'- end encoding ten functional proteins.

1.6 The life cycle of PVY

All (+) ssRNA viruses must follow same processes to replicate their genome (Sanfaçon, 2005). PVY infects a plant using an aphid vector, and enters the plant cell through the cytoplasm where the viral RNA is uncoated. The RNA

serves two purposes, it acts as a messenger (m) RNA that encodes viral protein and it is also used as a template for negative stranded RNA synthesis. The viral RNA undergo translation immediately after uncoating of the virus particles, by using the hosts' translational machinery to synthesize viral proteins. Some of these proteins such as the NIb and VPg are required for the virus genome replication and the initiation of the viral replication complex (VRC) which contains both viral and host proteins. The RNA-dependant RNA polymerase (RdRp) makes a copy of the positive sense RNA, using the VPg as a primer to create a negative sense copy of the genome, the strands are separated using the RNA helicase CI. The negative strand then acts as a template to synthesize more (+) ssRNA genomes. Newly synthesised RNA strands is either used as templates to generate more RNA strands, or used as mRNA to synthesize viral proteins or can be encapsidated to produce viral particles. This process is highly regulated to ensure large numbers of progeny RNA are produced. Further local cell-to-cell movement occurs within the infected leaf until the virus reaches the phloem vessels where it is transported with other macromolecules (i.e. photoassimilates nutrients) to other parts of the plant (Figure 1.2) including newly developed leaves, flowers, roots and tubers in potatoes.

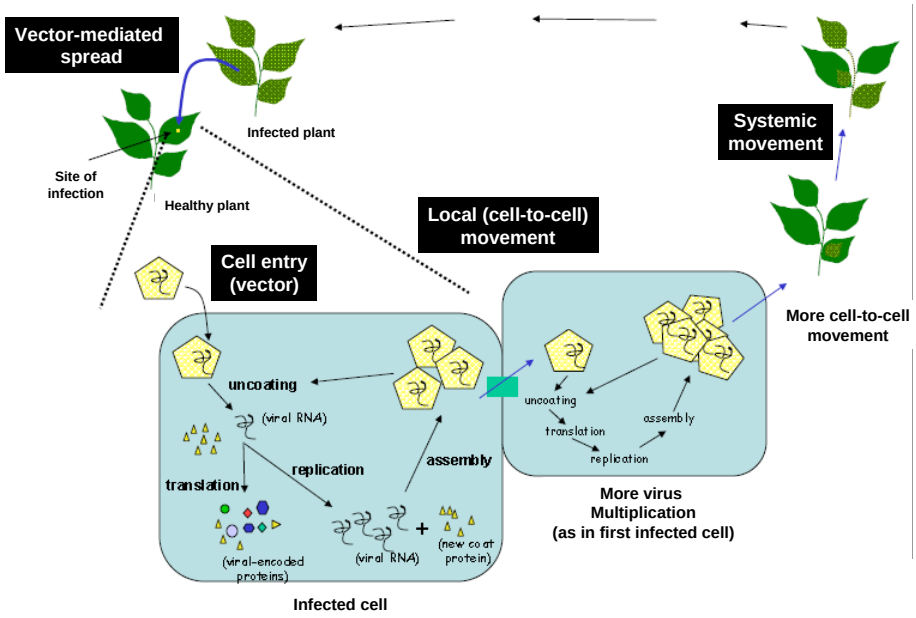


Figure 1.2 Different stages of plant virus life cycle. The infection of single cell, local cell-to-cell movement, systemic movement and transmission to a different plant. Virus particles are represented as a pentagon (Christophe Lacomme, SASA, personal communication).

1.7 PVY genome organization

The PVY genome is 9.7 kb in length of which the 9.1 kb open reading frame (ORF) encodes for approximately 3000 amino acids. The viral polyprotein is then cleaved into three virus encoded proteases (P1, HC-PRO, NIa-PRO) and further into ten products (Cuevas *et al.*, 2012). An additional peptide P3N-PIPO is translated from an overlapping ORF after +2 frameshifting of the P3. P3N-PIPO which encodes for a 77 amino acid protein (Dullemans *et al.*, 2011).

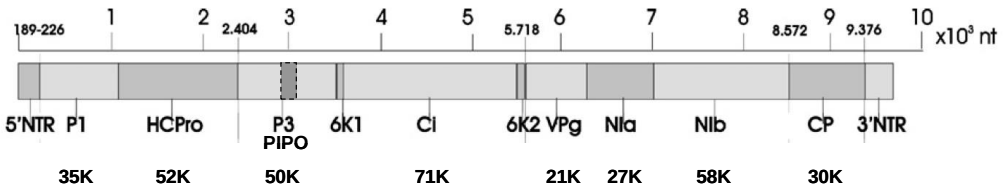


Figure 1.3 Genomic organization of PVY. The polyprotein is represented with the name of each protein generated after processing. P1, proteinase; HC-Pro, helper component-proteinase; P3, third protein, 6K1, 6-kDa protein 1; CI, helicase; 6K2, 6-kDa protein 2; V, VPg – viral genome-linked protein; P, NIa – proteinase; CP, coat protein.

P1, is a serine proteinase with a proteolytic activity which plays a role in PVY symptomatology and in promoting C-terminal proteolytic autocleavage. It is also used for RNA binding and host defense suppression (Rohozková & Navrátil, 2011). Although it is not needed for cell movement it does enhance the infectivity and movement of PVY within the host.

The HC-Pro (Helper Component Proteinase) is required for aphid transmission, systemic movement and is the main silencing suppressor for potyviruses (Kasschau & Carrington, 1998). HC-Pro binds with the involvement of a PTK motif in the C terminal third of its sequence to a DAG motif in the N terminal of the Coat protein (Blanc *et al.*, 1997). HC-Pro is implicated in the transmission of potyviruses acting as a link between the virus and the aphid mouth part (Wang *et al.*, 1996). HC-Pro also interacts with a range of host proteins and has been detected in chloroplasts of infected plants (Ala-Poikela *et al.* 2011). HC-Pro is believed to play an important role in the production of necrotic symptoms in tobacco (Tribodet *et al.*, 2005).

The P3 protein is required for PVY multiplication and is considered to be an important determinant in symptom development. The P3 protein gene shows little sequence homology among the potyvirus species and few functional motifs and structures have been identified in the resulting protein. P3 may be involved in viral amplification. The P3/CI complex may bring about wilting of the infected plant.

The role of the 6K1 has yet to be defined but its function might be similar to 6K2 as they are found in inclusion bodies during infection. Deletion of the 6K1 prevents proteolytic separation of the P3 from the Coat Protein (CP) (Merits *et al.*, 2002).

The CI protein (cylindrical-shaped inclusion body) is involved with cell to cell movement has Helicase-ATPase activity (Kerakainen *et al*., 2002; Carrington *et al.*, 1998) collects in cytoplasm, forms complexes which penetrate cell walls.

The protein 6K2 is involved in the anchoring of the viral replication complex to membranes.

NIa (the main protease) is involved in subcellular localization, protein-protein interactions. It is the primary protease required to cleave the viral polyprotein in to functional proteins (Gargouri-Bouzid *et al.*, 2006)

VPg is the virulence factor for PVY it is a multifunctional protein which is involved with multiple interactions between other virus proteins and the host plant. It is required for virus infectivity virus growth and replication, movement of the virus and interacts with viral RNA polymerase. Knocking

out of the VPg inhibits genome replication. Natural plant resistance to potyviruses are as a result of an inability for the VPg to interact with eukaryotic translocation initiation factor eIF4E within the plant (Grzela *et al.*, 2008).

NIb (Replicase) is responsible for genome replication and is a RNA-dependant RNA polymerase (Fellers *et al*., 1998).

The Coat Protein (CP) is also involved in aphid transmission, systemic movement and virus assembly (Urcuqui-Inchima *et al.*, 2001). The CP may be divided into three regions namely: the N-terminal, central and C-terminal regions. The variable N-terminal region carries the chief viral isolate specific epitopes. For this reason the CP gene of PVY has been used in the past to characterise isolates as being N, O, C or recombinant type. PVY CP is involved in several of the viral functions including regulation of RNA amplification, viral RNA encapsulation, movement of viral particles from cell to cell as well as systemic and aphid transmission (Bol 2008; Hofius *et al*.,2007; Shukla *et al*., 1994). The principle function of the CP is to encapsulate the viral genome. Approximately 2000 copies of CP monomers make up the non-enveloped filamentous structure which is specific to potyviruses.

Until recently it was believed that the PVY virus genome was a single open reading frame (ORF) than encoded for a 10 protein polypeptide. Chung *et al.* (2008) discovered a separate short open reading frame within the Turnip mosaic virus genome. They named this PIPO (Pretty Impressive *Potyviridae* ORF). In PVY the PIPO is 75-77 codons in length and does not act alone but

is expressed within the N-terminus of the P3 region and is therefore referred to as P3-PIPO it is believed to be involved with cell to cell movement as it is able to span cell walls and it is also thought to contribute towards symptom expression within the host plant.

1.8 PVY evolution

As for all other living organisms, variation in virus genomes can change to give rise to new types of strains to enable them to adapt to new and changing environmental conditions (Hull, 2002). Virus evolution is driven by the ever changing selective pressures from the environment (Hull, 2009). Sequence alterations such as insertions and deletions will often be lethal however maximising variation allows for the greatest level of adaptation. Variation of the virus genome allows the production of individuals that could eventually be better adapted to specific niches in which the virus has to establish successful infection in order to fulfill its life cycle.

(+) ss RNA viruses are characterised by large population sizes, fast replication and high mutation rates. Indeed, RNA viruses have highest rate of mutation of the genome of all organisms with an error rate of 10^{-4} to 10^{-6} mutations per base pair per generation (Sanjuán *et al.*, 2010) which results in a huge evolutionary potential. Two main forms of variation exist: mutation (changes in nucleotide) and recombination (exchange of blocks of genomic regions) (Hull, 2009). Mutations are most likely to occur during the replication of the RNA genome as a consequence of the lack of proof-reading activity of the viral-encoded RdRp (replicase) of the newly synthesized strand. With such a high error rate during replication RNA viruses populations can be described as

clouds or swarms of virus species progeny all with slightly different characteristics, the diversity of these species is sometimes referred to as quasispecies. The quasispecies concept could in part explain viral evolution (Holmes and Moya, 2002) , whereby broad spectrum of mutants can all be in competition with each other and subject to natural selection where limiting factors to variation include any mutations that are either lethal to replication or create an inability to package the virus. This means of rapid evolution can be advantageous for overcoming host defences or other selective pressures occurring during interactions between the virus with its host and vectors (Domingo *et al.*, 1998).

In RNA viruses, recombination is a major factor in their evolution (Roossinck, 1997). Three models for recombination exist, these are the replicase driven template switching model, RNA breakage and ligation model and breakage induced switching model. Recombination is common in potyviruses and is likely to impact on the evolution of PVY populations since most of the currently circulating isolates are believed to be recombinant forms (Cuevas *et al.*, 2012)**.** The template switching model suggest that recombination between two "donor" and "acceptor" viruses occurs by template switching at a region of homology between the two parental virus genomes. During replication of the donor RNA strand the polymerase switch strands in the homologous region, giving a recombinant RNA genome (Hull, 2009). The replicase-mediated template-switching model is considered to be the most likely mechanism for forming new RNA virus recombinants (Nagy and Simon, 1997). Recombination junctions are defined as regions where the sequence is likely to have switched from one virus strain type to another.

1.9 PVY strain nomenclature and characterisation

1.9.1. Biological characterisation of PVY

PVY exists as a complex of strains which can be distinguished on the basis of their biology (*i.e.* symptoms they elicit on indicator plants), serology and genome sequence.

The traditional method for classifying PVY was by using its biological properties, which is through examination of the symptoms elicited on plants (Singh *et al.*, 2008).

Using this approach PVY strains were generally divided into three major groups: the ordinary (PVY^{O}), the stipple streak strain (PVY^{C} leaf drop of potato) and PVY^{N} (veinal necrosis on tobacco). The hypersensitive response (HR: a form a resistance characterized by a localized cell death at infection sites) of potato cultivars harboring different resistant genes (*Nc*, *Ny* or *Nz*) to PVY strains together with the symptoms elicited on tobacco plants was traditionally used to define different PVY strains. . Using this approach PVY strains are currently classified into five distinct strain groups. These are PVY^{O}, PVY^{N}, PVY^{C}, PVY^{Z} and PVY^{E} (Table 2.1, Singh *et al.*, 2008; Kerlan *et al.*, 2011).

Table 1.3 Genetic background of the cultivars used for biological characterisation of PVY (Singh *et al.*, 2008). HR: hypersensitive response (leading to Resistance), S: susceptible (systemic movement and mosaic symptoms), VN: vein necrosis.

	PVY strain groups					
	PVY^C	**PVY^O**	**PVY^N**	**PVY^Z**	**PVY^E**	**Genetic background**
King Edward	**HR**	**S**	**S**	**S**	**S**	***Nc:ny:nz***
Pentland Crown **Desiree**	**S**	**HR**	**S**	**S**	**S**	***nc:Ny:nz***
Pentland Ivory **Maris Bard**	**HR**	**HR**	**S**	**HR**	**S**	***Nc:Ny:Nz***
Tobacco (White Burley)	**S**	**S**	**VN**	**S**	**S**	

1.9.2. Symptom expression and the importance of PVY strain definition

Different strains of PVY have different effects on the crop although the most significant effect is yield loss. There are several symptoms that can be observed within a potato crop including mild mosaic, sever mosaic, mottle, chlorosis and necrosis.

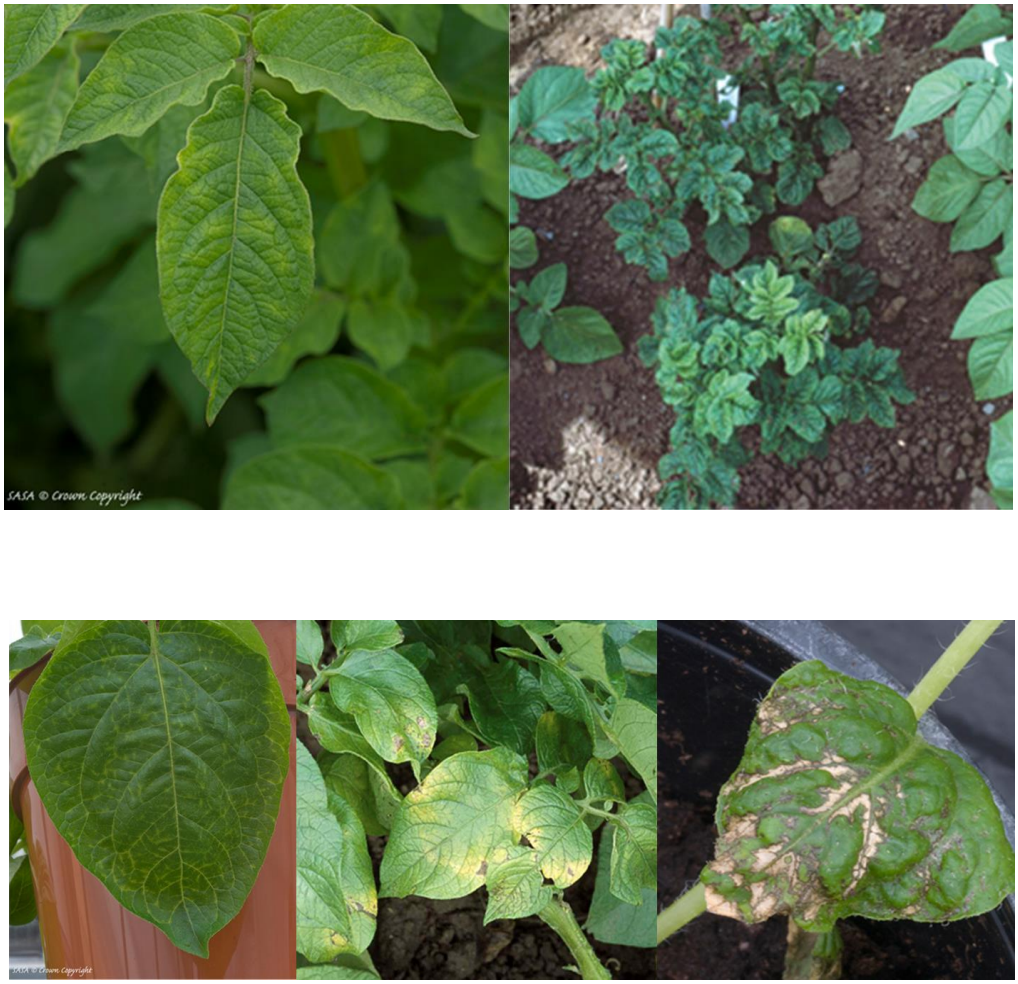


Figure 1.4 Symptoms induced by PVY in affected potato plants. Top left shows mild mosaic, top right shows a plant with severe mosaic, bottom left shows mottle on the leaf, bottom centre is chlorosis and bottom right shows necrosis.

PVY strains vary in the severity of disease they produce. Newly identified recombinant strains have the potential to cause substantial tuber losses for some varieties. It is therefore important to establish which strains are present in a country and what affect they are having on local cultivars.

PVY^O is known as the ordinary strain and was first identified by Smith (1931). It is known to cause a reduction in the yield of potatoes and produces severe mosaic symptoms in the plant. The symptoms induced by PVY^O in a potato crop include mosaic and mottling, chlorosis, leaf shed and premature death, dwarfing of plants, necrosis, mottling, chlorosis and leaf shed. The symptoms induced on potato cultivars by PVY^O are more severe than those initiated by PVY^N and are therefore affected plants are often considered to have severe mosaic.

PVY^C is related to the pepper strains of PVY and cause stipple streak which is a brittleness of the stems and petioles leading to leaf drop in the plant and identified by an HR response to *Nc*. Some PVY^C isolates are not transmissible by aphids (Blanco-Urgoiti, *et al.*, 1998).

PVY^N was first identified in the 1940s and 50s specifically because of its ability to produce vein necrosis in tobacco. In a tobacco crop it is capable of producing yield losses of 100% due to the necrosis (De Bokx & Huttinga, 1981). In potato it can be symptomless or produce weak mosaic in the foliage, however on discovery it was not known to produce tuber symptoms. It is defined by its ability to overcome HR responses in potato and is therefore able to infect a larger range of cultivars than PVY^O and PVY^C.

PVY^E is similar to PVY^N in that it has overcome *Ny*, *Nc* and *Nz* genes, which produce hypersensitive resistance in cultivars, however it does not produce vein necrosis in tobacco.

PVY^Z is also typified by its inability to produce vein necrosis but for this strain, cultivars known to harbour an *Nz* gene trigger a hypersensitive reaction when challenged with this strain. An example of this is isolate L26 (Kerlan *et al*., 2011) identified from North America which initiates an HR response in the cultivar Maris Bard.

In the past few decades new recombinant strains of the virus have emerged which are able to produce potato tuber necrotic ringspot disease (PTNRD) where circular necrotic lesions appear on the potato tubers which render them unmarketable. Some widely grown potato cultivars in Europe were found to be highly sensitive to PTNRD (Le Romancer and Nedellec, 1994, Van den Heuvel *et al.,* 1994). PTNRD was first recorded in 1980 in Hungary (Beczner *et al*., 1984) and spread across Europe in the 1980's and 1990's resulting in up to 100% of a crop harvest being destroyed. As a consequence of PTNRD epidemiology, almost all potato production was stopped in Slovenia and drastic changes in the varieties grown had to be adopted (Dolnièar, 2001). In Slovenia, the cultivar Igor was the most susceptible variety with over 50% of the tubers grown in some 18,000 ha affected by PTNRD (Kus, 1990). In Lebanon, the cultivar Lola was the most affected with 90% of 15,000 hectares grown damaged in 1989. Isolates that produced PTNRD in potatoes had the same biological and serological features as PVY^N and were therefore named PVY^{NTN} with the TN standing for tuber necrosis.

Figure 1.5 Potato Tuber Necrotic Ringspot Disease (PTNRD) symptoms shown in susceptible cultivar Nadine.

$PVY^{N\text{-}Wilga}$ was originally described as producing a symptomless infection (Glais *et al*., 2005) and therefore difficult to detect in the field due to the lack of symptoms it produces in the plant and it is now widespread throughout Europe. It can in some cultivars produce a systemic mottle and dwarfing, but it does not usually cause tuber necrosis (Kerlan *et al*., 2001), although some authors have reported PTNRD initiation with this strain (Piche, *et al.* 2004) As much as 90% of PVY isolates surveyed in Poland belong to the $PVY^{N\text{-}Wilga}$ group and cause significant yield reductions (Chrzanawska, 2001).

Along with all of the strains described, with PVY viruses subjected to high levels of mutation and recombination there are some isolates which do not fit neatly into any strain group. An example of this is isolate HR1 which elicits an HR reaction from Maris Bard which would suggest that is a PVY^{Z} however

PVY^{Z} should not elicit vein necrosis and this isolate does making it impossible to classify (Kerlan *et al*., 2011). The PVY virus is constantly adapting and changing and this will bring new characteristics and possibly new unforeseen problems to the industry as demonstrated by the appearance and spread of PTNRD.

1.9.3. Serological characterisation of PVY

In many laboratories, serological methods are often used for routine diagnosis of PVY using monoclonal antibodies due to their high throughput and cost effectiveness. Serological detection methods have been available since the 1930's but are now more refined and specific. Serological testing relies on a specific reaction between an antibody and its corresponding antigen in this case the PVY Coat Protein (Shukla & Ward, 1988).

Antibodies to the virus are generated from mammalian hosts for use in Enzyme Linked Immunosorbent Assay (ELISA). Monoclonal antibodies specific to epitopes within the CP region of the genome define the main serological groups PVY^{O}, PVY^{C} and PVY^{N} have been reported (Ounouna *et al*., 2002). However, recombinant $PVY^{N\text{-}Wilga}$ or $PVY^{N:O}$ variants react with the PVY^{O} antibodies while producing vein necrosis in tobacco, and therefore are considered to be biologically closer to PVY^{N} (Crosslin *et al*., 2005). It has been reported that a single amino acid substitution within the CP could lead to a misidentification of strain PVY^{O}-O5 by monoclonal antibodies (Chikh-Ali *et al*., 2008, Karasev *et al*., 2010). Polyclonal antibodies have been generated and recognize multiple epitopes from the CP allowing the diagnosis of all PVY

isolates without, however, distinguishing between different PVY strains (Ellis *et al.,* 1996). Serological testing using ELISA relies on enough virus load being present in the plant for detection, symptomatic leaves collected from field inspections will often have a high virus load. However for post-harvest testing it is often impossible to detect the virus infected but dormant tubers, as a result growing on tests are often required which take several weeks.

1.9.4 Molecular characterisation of PVY

PVY is a rapidly evolving virus, where both variation within strains and recombination events between strains occurs. Analysis of the genome using molecular biology methods has brought a new understanding of PVY at a nucleotidic level. Rapid developments in molecular biology and sequencing of isolates since the late 1980s have made it possible to characterise and classify PVY strains based on molecular characteristics (Thole *et al.*, 1993, Singh. 1999, Nie & Singh 2002, Rupar *et al.*, 2013) which may or may not correspond to traditional PVY strain groups. Overall, at the nucleotidic level, multiple isolates of the PVY^{O} and PVY^{N} groups differ from each other by approximately 8% along their genomes (Karasev *et al.,* 2010). Molecular genome characterization has identified where recombination events on PVY genomes have occurred to create new strains such as $PVY^{N\text{-}Wilga}$ and PVY^{NTN} (Boonham *et al.,* 2002). Molecular characterisation has also revealed that in many countries the majority of isolates are now recombinants between PVY^{O} and PVY^{N} (Yin *et al.,* 2012. Blanchard *et al.* 2008).

So far, it is not yet clear which molecular determinants of PVY are responsible for PTNRD. Glais *et al.* (2001) have suggested that the NIa and NIb and the CP might be involved. Several studies have associated a range of PVY strains (N-type, N-Wilga type, NTN-type) with PTNRD (for a review see Gray *et al*., 2010).There is a better understanding of the molecular determinants of vein necrosis, with some authors having reported specific nucleotide changes that relate to the initiation of vein necrosis in tobacco (Tribodet *et al.,* 2005; Rolland *et al.*, 2009; Faurez *et al*,. 2012). However these do not necessarily hold true in all cases (Hu *et al.*, 2009). Further surveys of PVY isolates originally in North-America identified isolates that cause PTNRD symptoms, but are molecularly distinct to the European PVY^{NTN} in that they have either none or only a single recombinant junction (Lorenzen, 2006). For this reason PVY isolated were separated in to $PVY^{EU\text{-}NTN}$ (European) or $PVY^{NA\text{-}NTN}$ (North-American). More recently $PVY^{NA\text{-}NTN}$ has been recorded from Europe and $PVY^{EU\text{-}NTN}$ in North-America, demonstrating that both strain types are now widespread globally.

Table 1. 4 Strains of PVY, identification depending on testing method

Proposed strain name (Singh *et al.,* 2008)	Biological strain group	Names given on basis of genome charateristics	Serological reaction SASA $PVY^{O/C}$ or SASA PVY^{N}	Vein necrosis tobacco
PVY^{O}	PVY^{O}	PVY^{O}	$PVY^{O/C}$	No
PVY^{N}	PVY^{N}	$PVY^{EU\text{-}N}$ $PVY^{NA\text{-}N}$	PVY^{N}	Yes
PVY^{NTN}	PVY^{N}	$PVY^{EU\text{-}NTN}$, $PVY^{NA\text{-}NTN}$	PVY^{N}	Yes
$PVY^{N\text{-}Wi}$	PVY^{N}	$PVY^{N\text{-}Wilga}$, $PVY^{N:O}$	$PVY^{O/C}$	Yes
PVY^{C}	PVY^{C}	PVY^{C1}, PVY^{C2}	$PVY^{O/C}$	No
PVY^{Z}	PVY^{Z}	$PVY^{EU\text{-}NTN}$	PVY^{N}	No
PVY^{E}	PVY^{E}	$PVY^{EU\text{-}NTN}$, $PVY^{NA\text{-}NTN}$	$PVY^{O/C}$	No

Table 1.4 above includes suggested nomenclature from Singh *et al*. 2008, biological strain group which is dependent on reaction of potato cultivars with

known resistance backgrounds, names given on the basis of sequence characteristics of the genome, serological identification using SASA specific PVY antibodies and finally whether vein necrosis is induced in tobacco.

Altogether, by using a combination of the biological, serological and molecular features of PVY isolates it should be possible to ascertain which mutations are important for symptom expression and virus fitness when relating to the host plant.

1.10 Epidemiology of PVY

The epidemiology of a virus encompasses all variable factors that influences the success and the spread of a virus (*i.e.* transmission, acquisition, reproduction). Variable factors affecting the crop will include differences in varietal susceptibility to virus, incidence of virus disease initially present in the crop, temperature, transmissibility and current disease prevention methods. Understanding the epidemiology of PVY is of critical importance to establish the reasons for the increase in the prominence of PVY^N. PVY is transmitted by two main methods, which is either plant to seed or plant to plant. Where the virus is translocated to the progeny tubers of a plant, any tubers produced by the next generation will be infected with PVY. All infected tubers will produce infected plants in the field, with consequently a much lower yield and this provides the main source of PVY inoculum. Cultural practices of identifying and removing symptomatic potatoes (roguing) is important in preventing the spread of the disease. Restricting the number of field generations of crops will also prevent the bulking up of virus inoculum. In Scotland field inspections identify symptomatic plants and strict tolerances for each crop grade are applied. Virus symptoms are separated into mild and severe mosaic. Infection with PVY^O triggers more severe leaf symptoms than PVY^N which have been reported in some cases to induce symptomless or only mild mosaic in the foliage (De Bokx & Huttinga, 1981, Gray *et al*., 2013). Weidemann (1988) suggests that differences in foliar symptom expression can be observed between different cultivars and on some occasions PVY^N can accumulate without being detected. There is the possibility that less aggressive PVY^N recombinant isolates might have been selected as a result of roguing with the

consequence that most symptomatic cases are identified and removed as opposed to milder symptoms triggered by PVY^N variants (Galvino-Costa *et al.,* 2012). Another possible factor facilitating the spread of PVY^N recombinant variants might be associated with the introduction of new potato cultivars expressing mild symptoms during PVY infection (Gray *et al*., 2013). As a relaxed tolerance for milder mosaic as opposed to severe mosaic symptoms applies, this could contribute to the selection of PVY^N infected material as opposed to PVY^O.

There are other sources of PVY inoculum, volunteer potatoes and neighbouring ware potato crops which potentially have a higher virus load act as reservoirs (Jones *et al.,* 1996). With PVY being able to infect many different plants within the *Solanacea* family there is the possibility that weeds surrounding potato crops could also be a source of inoculum.

1.11 Transmission of PVY

Living vectors of plant viruses include arthropods, nematodes and fungi. Most of these apart from beetles and fungi have needle type mouthparts that are involved with the uptake and ejection of virus particles. Aphids are amongst the most common and effective virus vectors due to their high numbers, migratory patterns and probing mouthparts (Hooks and Fereres, 2006). The short aphid lifecycle can occur repeatedly throughout the life of the host plant. Winged migrants are broadly responsible for virus spread within crops (Robert *et al.*, 2000). Production of these morphs may be controlled by many factors including overcrowding, the presence of predators and environmental cues (Mehrparvar *et al.*, 2013). Aphids are found worldwide though they are most

predominantly in temperate regions of the Northern Hemisphere (Blackman and Eastop, 1984) and as such are particularly important in the United Kingdom. Viruses can be categorised into two main groups, according to the mode of aphid-transmission involved, either persistent (circulative) or non-persistent (non-circulative) (Ng and Perry, 2004, Brault *et al.*, 2010). Persistently transmitted viruses such as PLRV enter the aphid's circulatory system and can remain viable for the lifetime of the aphid, the success of the virus in this group depend on aphid species remaining within the crop (Kotzampigikis *et al.*, 2010).

PVY is transmitted in a non-persistent or non-circulative manner. Non-persistently transmitted viruses attach to the stylet of the aphid which means that the aphid will acquire the virus when probing a plant and will transmit it in the next probing episodes (Radcliffe & Lagnaoui, 2007). In non-circulative transmission the virus is retained wihin the stylet before deposition, the virus is usually only retained for less than an hour (Peters, 1987) but in some winged forms can remain for up to 17 hours (Kostiw, 1975). Moulting abolishes transmission which suggests that virons bind to the outer cuticle (Murant, 1975). As a result of this method of transmission it is well accepted that non-colonising aphids such as cereal aphids, which do not feed on the potato are able to transmit the virus (Sigvald, 2008). Over 40 species of aphid are known to be able to transmit PVY (Edwardson and Christie, 1997; Quenouille *et al.*, 2013). The UK has over 500 species present and the species thought to be responsible for the greatest levels of PVY transmission are non-colonising species. The interaction between PVY and its aphid vectors is

highly complex. Although 40 species have been identified as being able to transmit PVY the number is perhaps greater. *Myzus persicae* (Peach potato aphid) is known to be the most effective transmitter of PVY. However, in Scotland the two cereal aphid species *Metopolophium dirhodum* (Rose Grain aphid) and *Sitobion avenae* (Grain aphid) are thought to be responsible for 71% of PVY transmission (Pickup *et al*., 2009).

With PVY^N becoming more dominant than PVY^O it is possible that certain aphids are more efficient in spreading PVY^N than PVY^O and this could be driving changes in the PVY population. During transmission the virus-encoded factor helper-component protease (HC-Pro) is known to be essential for aphid virus transmission and the lack of this viral protein prevents binding. The helper component for potyviruses is reported to interact with many host proteins and acts by regulating virus uptake, binding the virus to the receptor sites in the aphid food canal (Maia *et al.*, 1996). The HC-Pro is a viral nonstructural protein required for transmission proposed by Govier and Kassanis (1974) who suggested that the HC-Pro works as a bifunctional molecule by joining the virus particles with putative receptors on the aphid's stylet (so-called "bridge hypothesis"; Pirone and Blanc, 1996). The HC-Pro and the CP interact and act as a molecular bridge, mediating the reversible retention of virions to uncharacterised binding sites in the vector mouthparts (Fernandes-Calvino *et al.*, 2010). The HC-Pro must be acquired prior to or with the virus (Pirone and Thornbury, 1984). The HC-Pro present in PVY can also be utilised *in trans* as an heterologous helper by other viruses *such as Potato aucuba mosaic virus* to enable transmission, which can therefore only be transmitted along with PVY (Manoussopoulos JN, 2000).

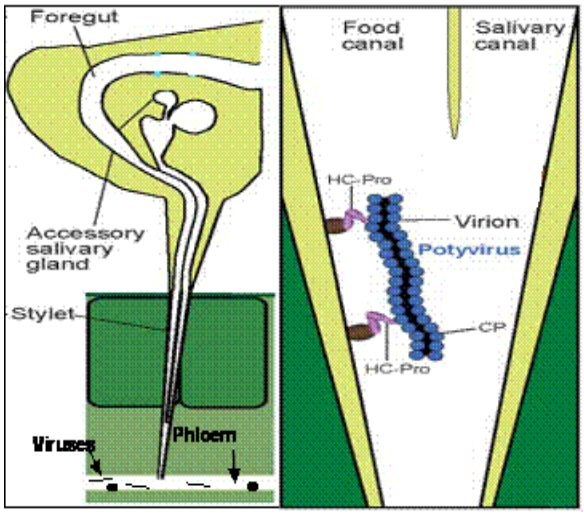


Figure 1.6 Aphid stylet showing feeding method and virus attachment adapted from Ng and Falk (2006)

With PVY^N replacing PVY^O in Scotland it will be important to understand what mechanisms are responsible for this shift. The HC-Pro and the Coat Protein (CP) region of the PVY genome both contain areas of recombination. Differences in amino acid composition of the HC-Pro region and the CP region could therefore account for differences in effectiveness of transmission of different strains of PVY.

1.12 Control of PVY

1.12.1. Genetic resistance to infection: Extreme Resistance (ER) and Hypersensitive Response (HR)

Plants have developed a wide array of resistance mechanisms to PVY. Amongst them, are resistance genes which can either produce a Hypersensitive Response (HR) or Extreme Resistance (ER), dominant genes code for both types of response to virus infection (Tain & Valkonen, 2013; Hamalainen *et al*., 2000). HR is strain specific and is conferred by plant resistance (R) genes that enable the recognition of a corresponding avirulence gene from the invading pathogen (Mihovilovich *et al*., 1998). In response to PVY^{O} infection, plants carrying an *Ny* gene will produce a hypersensitive response with local necrotic lesions through cell collapse and cell death to prevent the spread of a pathogen. HR effectiveness to PVY appears to be affected by environmental factors such as temperature and aphid numbers (Robert *et al*., 2000; Szajko *et al*., 2008). When inoculation pressure is high the plant can become systemically infected rather than producing a hypersensitive response. Salanki *et al*. (2007) suggest that this may be due to competition between the HR and the virus infection pathways and if activation of HR is delayed this can lead to suppression of the HR and systemic infection of the plant. Generally HR genes do not provide sufficient protection against PVY but are useful in the biological characterisation of strain groups and are used to discriminate PVY strains in their potato hosts (see Section 1.5.1 above). Resistance genes conferring Extreme Resistance (ER) have already been introduced to some European potato cultivars. The *Ry* genes for ER confer extremely high level of

protection against different strains of PVY (Ross 1986; Valkonen *et al.*. 1996). Three *Ry* genes originally introgressed from wild diploid *Solanum* species have been localized by molecular mapping on tetraploid *S. tuberosum* chromosomes (reviewed by Szajko *et al.*, 2008): Ry_{sto} (designated also as $Ry\text{-}f_{sto}$) derived from *S. stoloniferum*, Ry_{adg} from *S. tuberosum* ssp. *andigena* and the gene Ry_{chc} derived from *S. chacoense*. While ER genes are highly effective in controlling all strains of the virus, cultivars harbouring these genes can exhibit male sterility, which limits their usefulness in breeding programs (Szajko *et al.*, 2008). Le Romancer and Nedellec (1997) emphasized that even if the cultivars that carry a *Ry* gene are field resistant this will not be sufficient to decrease the PVY^{NTN} population because of its capacity to affect other *Solanaceous* hosts. As the PVY genome is highly variable, any monogenic resistance bred in to potato cultivars could lead to further selection of new PVY variants that will overcome this resistance. It may be that plant breeding in itself has a role to play in the emergence of new PVY variants. PVY^{O} has affected potato crops for over 80 years and for this reason, potato cultivars have been bred with a level of resistance to this strain of the disease. However, this may have inadvertently selected for PVY^{N} and their recombinants. Breeding in resistance to PVY^{N} is far more challenging and this may in part explain the current prevalence of PVY^{N} in potato crops.

1.12.2. Mature plant resistance

Mature plant resistance (MPR) is a broad spectrum resistance mechanism where potato plants inoculated late in the growing season with viruses such as PVY display increased resistance to PVY infection and are less likely to have

infected progeny tubers (Gibson, 1991; Sigvald, 1985). Basky (2002) found that early season aphid vector pressure was critical in determining the level of virus spread; the reason for this could be that older plants become less susceptible to PVY as mature plant resistance develops. Some level of control of aphid-transmitted viruses can therefore be achieved by early planting of potato crops to reach maturity before high aphid pressure could be observed. Measures to control virus spread are likely to be most effective when crops are most susceptible, knowledge of MPR development is an important element in forecasting spread of viruses in potato crops (Gibson, 1991).

1.12.3. Control and management of aphid vectors.

Aphid monitoring (using suction traps or yellow water traps in different locations) is used for aphid and virus forecasting. Virus control programmes normally start after aphid are caught in the traps. Controlling aphids colonising a potato crop (*Myzus persicae*, *Macrosiphon euphorbiae*) by applying insecticides has proven to be an inefficient way to prevent PVY spread. Previous research indicates non-colonizing aphid species are also important in the spread of PVY (in particular *R. padi*, *M. dirhodum*, and *B. helechrysi*) (Boquel et al, 2013; Derron and Goy, 1990). As PVY is transmitted non-persistently by non-colonizing aphids, interference with virus acquisition can be achieved by spraying mineral oil (for a review see Al-Mrabeh *et al.*, 2010). This approach can decrease PVY transmission by about 20% up to 60% in some cases (Weidemann, 1988; Al-Mrabeh *et al.*, 2010). Other methods can be grouped as "cultural control" approaches which aim to reduce the impact of aphids by using ground coverings (mulches), intercropping; preventing aphid

access by physically protecting crops or enhancing aphid mortality by providing habitats for aphid predators (Wratten *et al.*, 2007).

Understanding the biological and molecular features of PVY present in Scotland and incorporating this with epidemiological studies of the virus will be of critical importance in establishing a greater understanding of the disease which could lead to more effective control.

1.13 Aims and Objectives of Project

It is anticipated that PVY recombinants are prevalent in most, if not all, potato-growing areas including the UK (Barker *et al.*, 2009). This emphasises the fact that PVY population dynamics need to be monitored not only at the serological but also at the molecular level to obtain a more exhaustive knowledge of PVY population dynamics and ultimately breed and select for potato varieties that display resistance to the most prevalent PVY variants. So far, no detailed studies on the nature of PVY isolates found in field conditions in Scottish seed potatoes, and the UK as a whole, have been undertaken. The ability to distinguish between different strains of the virus is a pre-requisite to the understanding of PVY^N population dynamics, evolution and pathogenicity. The objectives of this research is to address the following:

1.13.1 What is the nature of PVY^N isolates in Scotland?

This will be established by using PVY isolates identified by the virology laboratory in 2009 as being serologically PVY^N.

- The symptoms that each isolate produces on tobacco indicator plants is to be recorded.

- Characterisation of the molecular nature of PVY^N isolates intercepted in Scottish seed potato crops through targeted partial sequencing of known recombinant junctions (RJs) identified along the PVY genome.

- Full-length sequencing of a relevant subset of PVY variants will be undertaken to refine the analysis of their genetic background.

- Along with the above, a serological survey will be completed of all symptomatic leaf samples taken during the 2010 growing crop season inspections. Both monoclonal and polyclonal antibodies will be used to identify any differences between antibody detection and maximize the chances of intercepting a wider range of PVY variants.

1.13.2 What risks do $PVY^{N/NTN}$ variants pose to the seed potato industry and which varieties are most affected?

In order to establish the risks that PVY^{N} variants pose to the Scottish seed potato industry it is important to understand the symptoms they produce and also whether PTNRD could affect Scottish cultivars. Therefore:

- A selection of isolates that are found to be representative of the diversity of PVY^{N} population in the field will further be biologically characterised using a range of potato cultivars of known resistance to PVY with the view to characterise their biotype (Singh *et al*., 2008).

- The severity of foliar mosaic symptoms will be observed for a selection of cultivars and isolates and this will be evaluated against virus titre.

- Potato Tuber Necrotic Ringspot Disease (PTNRD) symptom development will also be evaluated in infected tuber progeny in order to assess the potential link between foliar symptom severity, the molecular-biological PVY isolate type and tuber symptoms.

- Tubers for one trial will be grown on over 3 generations to establish if there is a link between PTNRD symptom severity and generational infection.

- A range of the most commonly grown cultivars in Scotland will be assessed for PTNRD symptoms induced by Scottish PVY isolates, to determine if PTNRD could be a threat to the Scottish potato industry.

1.13.3 What drives PVY biodiversity?

Aspects of the epidemiology and fitness of PVY variants will be investigated, in order to try and establish the reasons behind the increase in PVY^{N} in Scotland. This work has been integrated with an on-going epidemiology project investigating the spread of PVY in a field situation throughout the growing season at SASA for the past 10 years.

- Aphid transmissibility of 3 different PVY isolates will be assessed through the use of *Nicotiana debneyi* plants, which are replaced on a weekly basis throughout a growing season and tested for PVY. Relating these results to the aphid vector pressure should give an indication of whether different PVY isolates are transmitted in the

same frequency and at the same time, and should also give an indication of whether the same aphid species are involved.

- Maris Piper potato bait plants from the field trial will be harvested to assess the level of virus infection within the tubers following a growing season, this should give a good indication of which isolates are more effective at infecting tubers and therefore successfully provide a source of infection for the next generation.

- To assess within plant competition of isolates, Maris Piper plants will be infected with more than one isolate of PVY in glasshouse conditions and the level of infection by each isolate in the progeny tubers will be assessed.

Chapter 2: Serological and Molecular characterisation of Scottish PVY isolates

2.1 Introduction

Identification of PVY isolates according to strain type was traditionally achieved using plants as biological indicators (Jones, 1990). Due to the ability for high throughput and cost effectiveness serological testing with Double Antibody Sandwich Enzyme Linked Immunosorbent Assay (DAS - ELISA) is regularly used for routine diagnostics. DAS-ELISA has limitations in strain determination of PVY isolates as it requires the use of monoclonal antibodies. The type of monoclonal antibodies used for serological testing allows for the discrimination of isolates as PVY^{N} or $PVY^{O/C}$ types, on the basis on specific motifs within the coat protein region of the genome (Boonham and Barker, 1998; Ounouna *et al.*, 2002). Recently serological testing using SASA PVY^{N} monoclonal antibodies has been shown to miss some South American isolates (Galvino-Costa *et al.*, 2012). For this reason and to uncover all diversity in Scottish PVY isolates a survey was undertaken of all symptomatic leaf samples submitted to the virology laboratory in 2009 using polyclonal antibodies. Polyclonal antibodies are able to use a range of epitopes rather than a single one (Shukla *et al.* 1989; Ellis *et al.*, 1996) to identify a virus as belonging to PVY but they are not able to distinguish between strains. Conducting a polyclonal survey will allow for any symptom inducing PVY isolates that are missed by SASA PVY^{N} or $PVY^{O/C}$ to be identified and subsequently be characterised through genome analysis. Limitations of

serological testing are that only PVY^{N} and $PVY^{O/C}$ can be discriminated between and with recombinant isolates becoming increasingly dominant new methods are now required to distinguish strains further and more accurately.

The development of molecular technologies has advanced the diagnostics of PVY and lead to characterisation at the nucleotidic level. These methods include, RT-PCR (Ghosh and Bapat, 2006) quantitative RT-PCR (Rupar *et al.*, 2013), multiplex reactions (Lorenzen, 2006), microarrays (Boonham, 2003) cDNA hybridisation (Hopp *et al.*, 1988) and full length (Dullemans *et al.*, 2011) and partial sequencing of the genome (Hay *et al.*, 1989; Li *et al.*, 2006). From sequencing and recombinant junction analysis it is possible to determine where variation occurs between isolates and where recombination events have occurred (Nie and Singh, 2002). Figure 2.1 shows the molecular nature of the defined strains of PVY (in accordance with Schubert *et al*., 2007). Highlighted are the regions where recombination events have predominantly occurred (recombinant junction R1, R2 and R3) between PVY type O, N, C, NA strains. Previous work on the sequencing and biological characterization of PVY field isolates around the world have demonstrated that PVY recombinant variants are widespread worldwide (Visser *et al.*, 2012; Hu *et al.*, 2009a; Yin *et al.* 2012) and can also be found in Scottish seed potatoes (Barker *et al.*, 2009).

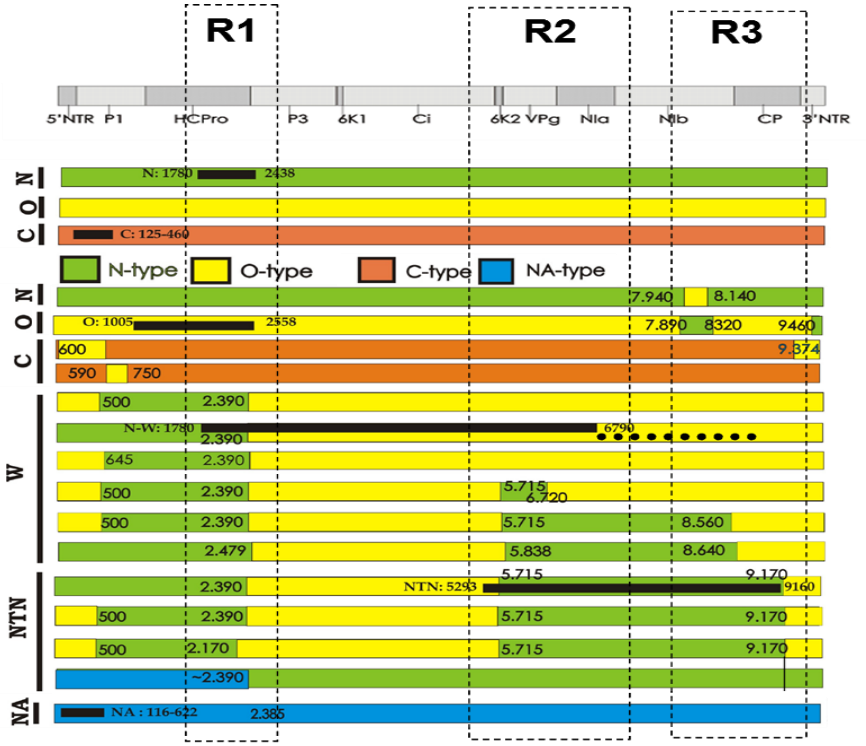


Figure 2.1: Genomic structure of isolates of different PVY strains and variants. The recombination points are shown inside the genomes. Recombinant junctions R1, R2 and R3 selected for further partial sequencing are boxed (the PVY type strains are color-coded: green N-type, yellow O-type, orange C-type, blue North-American NA- type strain).

The overall aim of the study is to identify which molecular groups (variants) and PVY strains are prevalent within the PVY^N serotype in Scotland. Firstly, targeted sequencing on Recombinant Junctions (RJs) previously identified in the PVY genomes (Nie and Singh, 2003, Lorenzen *et al.*, 2006) will be undertaken and assessed to confirm its potential usefulness to discriminate between PVY molecular variants and identify the relative molecular diversity of PVY variants within these regions and the molecular diversity of individual viral proteins. This approach will help to identify PVY genome portions of

high/low variability and potentially under selective pressure. Further, full length sequencing of a representative selection of PVY isolate belonging to the previously identified molecular groups will be carried out to confirm their molecular nature and as well to assess the published necrotic determinants of tobacco vein necrosis and whether the same or additional genetic determinants are involved in potato tuber necrotic ringspot disease (PTNRD) development. The latter will be undertaken in conjunction with the studies on pathogenicity of the same selected PVY molecular variants addressed in Chapter 3.

For this purpose the genomic sequence from representative PVY strains were retrieved from public database (GenBank) and included in phylogenetic analysis to define groupings of PVY^{N} field isolates on the basis of sequence homologies. All isolates will be grouped on the basis of sequence homologies within these three regions (R1-R2-R3) that encompass major recombination hot-spots and provide the highest nucleotidic sequence variations between PVY strains.

2.2. Methods

2.2.1. Sampling, storage and infection of PVY^N isolates.

Prior to the start of this project, virology laboratory staff collected all PVY positive samples submitted by RPID (Rural Payment Inspection Directorate, Scottish Government) field inspectors from the 2009 season which were identified by DAS-ELISA. They inoculated these isolates in to tobacco plants *Nicotiana tabacum* cv. White Burley and *Nicotiana benthamiana*. Leaves of these plants were freeze dried using an Edwards Modulyo 4K freeze-dryer (Crawley, UK) labelled and stored for use. The isolates that were used for the following work were only those identified as PVY^N by ELISA. Plant sap was obtained following grinding of fresh or lyphohilised leaf material in Sorensens phosphate buffer) using a homex grinder (Bioreba, Nyon, Switzerland). A further 5mL of leaf extraction buffer was added to the bag after maceration. Plants were re-inoculated for biological characterisation as described in chapter 2.

2.2.2. Double Antibody Sandwich - Enzyme-Linked Immunosorbent assay (DAS-ELISA).

Double antibody sandwich (DAS) ELISA was used for serological differentiation of PVY isolates was performed as previously described (Clark and Adams, 1977; Barker 1997).

http://www.q-bank.eu/Virus/LocalFiles/Protocol%20DAS-ELISA%20virus.pdf. All leaf samples collected from field inspections that

were submitted to the SASA Virology laboratory were tested using Polyclonal (SASA PAb PVY) and Monoclonal (SASA MAb $PVY^{O/C}$, SASA MAb PVY^{N}) antibodies. All antibodies available from SASA are generated by SASA and are raised through inoculating mice with typical virus isolates. They are validated by the virology laboratory who carry out testing for the Scottish seed potato classification scheme. SASA has not published the production of these antibodies. The binding sites for SASA MAb PVY^{N} have been identified as a linear epitope spanning the amino acids 22-30 within the N-terminal of the capsid protein (Nikolaeva *et al.*, 2012). SASA MAb PVY^{N} is specific to PVY^{N} and PVY^{NTN}, while SASA MAb PVY^{O} is specific to PVY^{O}, $PVY^{N\text{-}Wilga}$ and PVY^{C} strains (Karasev *et al.*, 2010). For DAS-ELISA detection of $PVY^{O/C}$ and PVY^{N}, polystyrene microtitre 96-well plates (NUNC) were coated with (200µL of 1:1000 dilution). The plate was loaded with 200µL of plant sap and incubated for 16hours at 5°C before being washed with 1xPBST buffer (containing 3.2 mM Na2, HPO4, 0.5 mM KH2PO41.3 mM KCl, 135 mM NaCl, 0.05% Tween, pH 7.4). Samples were conjugated with either $PVY^{O/C}$ MAb or PVY^{N} MAb (200µL of 1:4000 dilution). For DAS-ELISA with PVY polyclonal antibodies (PAb), plates were coated and conjugated with SASA PVY PAb (200µL of 1:8000 dilution), incubated at 33°C for 2 hours and washed with PBST buffer. The absorbance was measured after 1 hour of incubation with the substrate buffer (Paranitrophenyl phosphate) at room temperature. Absorbance was measured at OD_{405nm} using a Sunrise microplate absorbance reader (Tecan, Mannedof, Switzerland). A sample was considered to be positive if the OD_{405nm} value is twice more than the OD_{405nm} absorbance from a healthy non-infected control plant.

2.2.3. RNA extraction.

Total RNA was extracted from symptomatic leaves of tobacco plants inoculated by selected field isolates using the MagExtractor-RNA Kit (Toyobo, Japan) performed on the magnetic particle processor KingFisher mL (Thermo Scientific, Basingstoke, UK) and RNA was stored at -20°C for future use as previously described (Boonham *et al.*, 2008; Mortensen *et al.*, 2010).

2.2.4. PCR Amplification of PVY R1, R2 and R3 regions.

Reverse Transcription (RT)-PCR was performed in one step using 2x Jumpstart REDTaq Ready Mix (Sigma, UK) using 2µL of RNA, 0.05µL of 100µM primers (see Table 2.1 for primers list) (250nM final concentration) and 0.05µL of Moloney murine leukaemia virus reverse transcriptase (Promega, Southampton UK) in 20µL total volume. Conditions for the RT-PCR are as follows: 48ºC for 30 min, 95ºC for 5 min and 35 cycles of 94°C for 3 sec, 55°C for 30 sec, 54°C for 30 sec, 72°C for 1 min followed by 72°C for 7 min, performed on an MJ Research Peltier Thermal Cycler PTC-200 (Waltham, UK).

The size of PCR fragments was measured on 1% agarose gel stained with Gel Red (Biotium, UK) and photographed under UV illumination. Primers for cDNA synthesis and sequencing were designed from various PVY genomes available in GenBank (Table 2.2) using SeqBuilder software from Lasergene core suite (DNASTAR, Madison)

Table 2.1 Primers selected for PCR and sequencing

Primer	Sequence (5'-3')	Nucleotide position
PCR1-FWD	ATAAGAAAATCAACGCAAAAACAC	24
PCR1-REV	CGACCCACGCACTATGAAT	972
PCR2-2FWD	GCGCAGCAAAAACATA	582
PCR2-2REV	AGCGCTTGAGAACTGAACCATTG	1050
PCR3-2FWD	ATCACATCGCGCAGTCGTT	735
PCR3-2REV	GGGCACAGGTAGGGCAGGTTAT	1213
PCR-2FWD	GGTCGCACGAAGGAAAAATCTATG	968
PCR2-REV	CCTTCGAGCTCGTGCACTTCTTAC	1865
PCR4-2FWD	TGGGCACAAATGAGATA	1081
PCR4-2REV	GCTTTGGCAGATAGTTTA	1501
HCPro-F1	AAGTATGGTTTAAATCGATTG	1269
HCPro-R1	ATATTTTCATTTGAGCACAAG	2218
PVYR1FWD	**GARATGTTATAYATTGCCARGCAR**	**2035**
PVYR1REV	**CTRTGGGTTTTATGWACYGAGTGATAGAG**	**3043**
PCR3-FWD	GTCAGCGGCAGAAACACTCGTC	2709
PCR3-REV	GCCATAAATGCTACCAC	3554
PCR5-2FWD	CTATTCAAGGCGGGTTTTC	2869
PCR5-2REV	ACTATCTGAGGGTTTACTGACTT	3458
PCR6-2FWD	TAGTATGTTAACCAGCGTAGTG	3255
PCR6-2REV	AAGTGCCCCTCGGTTCTGTAAT	3833
PCR7-2FWD	AAAGGAGTGATTGCGTGTTC	3581
PCR7-2REV	ATTGAGAGCGATTATTGG	4153
PCR5-FWD	ACAATTMRAACATCATCAGTG	3730
PCR5-REV	TTMARCCCAAAATCTACAACY	4634
PCR6-FWD	TTRGCYAAGCTYCTAACR	4450
PCR6-REV	YTCCTCYTCRAKCARTCTYT	5325
PCR7-FWD	CTYGGAGTGGYYTTRGAMA	5095
PCR7-REV	CAACGAACTGGATGAATGA	5950
PVYR2FWD	**YGCMATYCCMAGAACYCTAA**	**5277**
PVYR2-2F	**GGGCTGGCTTTGAAATTGA**	**5777**
PVYR2-2R	**AAACCTGTGATGAGTGATTTGGCTTCATG**	**6189**
PVY-R2REV	**TMGTRCTYGTTTCTGTGATGATYGAYG**	**6673**
PCR8-FWD	AATGTATGGGTTCGGTTTY	6375
PCR8-REV	GAGTATTTCATTATGTCCTTTAT	7244
PCR9-FWD	GTGGCGACAATGAAGAGT	7066
PCR9-REV	CTGGAGTTGAGATTGGYGT	7906
R3F-7749	**CGATGCCGATGGTTCACAAT**	**7749**
R3R-8940	**CCGTTGATGTTTGGCGAGGTT**	**8378**
PVYF8378	**KCAACRATAGCGCAGGAAGG**	**8940**
PVYR 9594	**GCTACGACAGAATCGCAACA**	**9594**

Primers marked in bold print are those used for partial sequencing, Figure 2.2 shows the location of the primers along the genome.

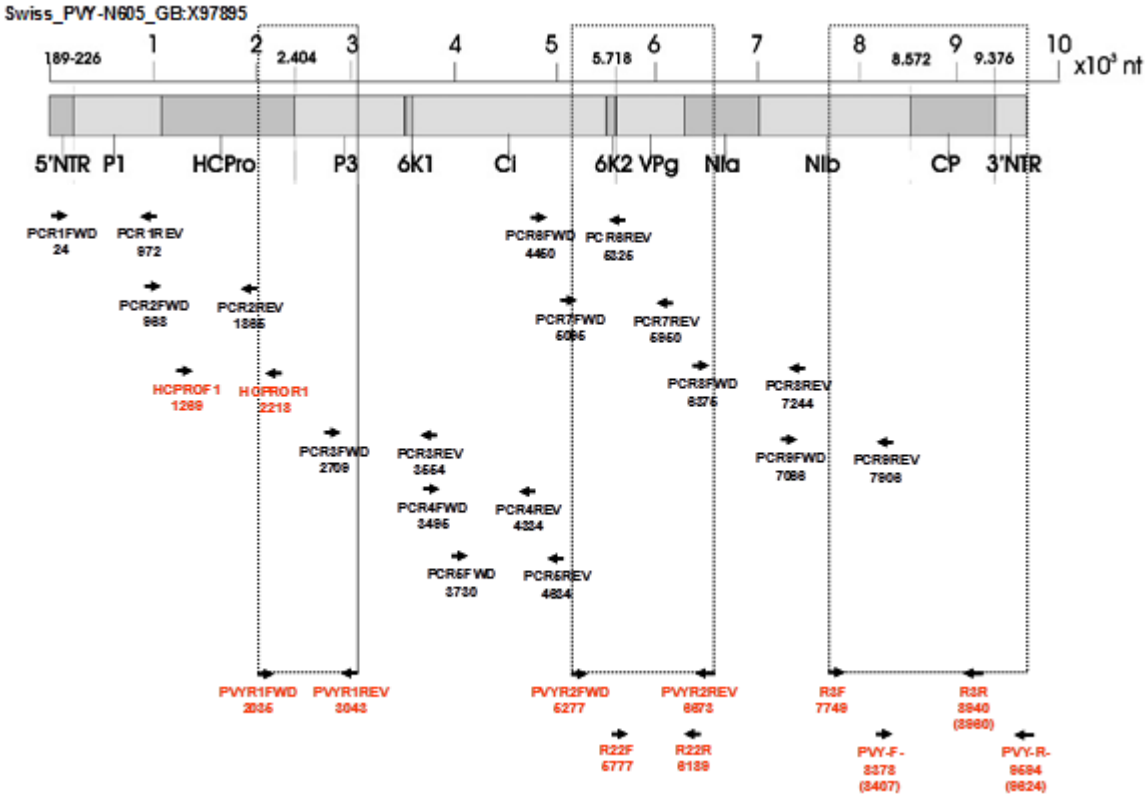


Figure 2.2 Positions of the primers used for the sequencing of recombinant junctions. Primers for R1-R2-R3 are labelled in red and the remaining primers were used for whole genome sequencing of PVY isolates as detailed in Table 2.1.

2.2.6. Sequencing

Prior to sequencing, the PCR product was treated using a 1 µL aliquot of Exonuclease III and Shrimp Alkaline Phosphatase (EXOSAP) master mix (Affymetrix UK ltd, High Wycombe) containing 1µL of Exonuclease III and 1 µL SAP mastermix was added to each PCR reaction, homogenised, then incubated at 37 ^{o}C for 45 mins followed by heat inactivation at 80 ^{o}C for 15 mins and stored at 10 ^{o}C until use. The products of the RT-PCR were then prepared for sequencing using the Big Dye Terminator Cycle Sequencing version 3.1 Kit (Applied Biosystems). The sequencing reaction included 1.75µL of 5X Sequencing buffer, 0.5µL of Big Dye Terminator, 1 µL of Primers (3nM final concentration), 6.5µL of H_2O and 1µL of the RT-PCR product (final volume 10 µL). Sequence reactions were performed using the primers used in the RT-PCR (listed in Table 2.1) Conditions for the sequencing reaction are as follows: 96^{o}C for 1min and 25 cycles of 96 ^{o}C for 10 sec, 50 ^{o}C for 5 sec, 60 ^{o}C for 4 mins followed by 4^{o}C indefinitely. The sequencing reactions were run on a 3130xl Genetic Analyser (Applied Biosystems). Sequences were assembled using the SeqMan Pro module of Lasergene version 8 (DNAStar, Madison, WI, USA). Relationships between sequences were visualised by the creations of Neighbour-Joining trees using the algorithm of Saitou and Nei (1987) implemented in MEGA 5 (Tamura *et al.*, 2011). Sequences from the PVY isolates were compared against previously identified sequences from Scotland and other countries from GenBank, detailed in Table 2.2.

Table 2.2 Whole genome PVY reference sequences retrieved from GenBank

Isolate	GenBank accession number	Published country of origin	Classification
Hungarian NTN	M95491	Hungary	NTN
L26	FJ204165	USA	NTN/E
Nicola	AJ890346	Germany	N
N605	X97895	Switzerland	N
New Zealand	AM268435	South Island, New Zealand	N
SASA 61	AJ585198	Scotland, UK	O according to genbank
RRA 1	AY884984	USA	NA?
SCRI N	AJ585197	Scotland, UK	N
SASA 110	AJ585195	Scotland, UK	O
SCRI O	AJ585196	Scotland, UK	O
L56	AY745492	Canada	N-Wilga
SASA 207	AJ584851	Scotland, UK	N

SASA 61, SCRI N, SASA 110, SCRI O and SASA 207 (Barker *et al*.2009); Nicola and New Zealand (Schubert *et al*., 2007); L26 (Thole *et al.*, 1993); L26 (Hu *et al*., 2009b); L56 (Nie *et al*., 2004); RRA 1 (Alabi *et al*., 2012); N605 (Jakab *et al*., 1997).

2.2.7. Phylogenetic analysis of PVY isolates by targeted sequencing of recombinant junctions

Phylogenetic analysis and amino acid relationships were calculated using MEGA 5 (Tamura *et al.*, 2011). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) using nucleotides and the Tamura Nei model (1993). Bootstrap consensus trees were inferred from 2000 replicates and taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 70% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are

shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Tamura-Nei method (1993) and are in the units of the number of base substitutions per site. The analysis involved 98 nucleotide sequences. Codon positions included were 1st, 2nd, 3rd, Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).Where data was missing from the ends of nucleotide sequence these ends were trimmed in order to include as many isolates as possible, for individual isolates with a lot of data missing these isolates were removed in order that a full comparison between isolates could be achieved.

2.2.8. Whole genome phylogenetic analysis of a selection of PVY isolates

A selection of Scottish field isolates were chosen for full length sequencing on the basis of their groupings in the R1, R2 and R3 regions. In total 10 isolates were selected including 7 field isolates and three disease variety (DV). Isolates were selected for full length sequencing on the basis of the sequence characteristics of the R1, R2 and R3 regions was undertaken for most PVY isolates used in the pathogenicity trials described in Chapter 3.

The isolates used for full length sequencing were PVY^{E}: 10088 $PVY^{EU\text{-}NTN}$: 10057, DV76 and 9681 PVY^{N}: 9552 $PVY^{NA\text{-}NTN}$: 9737 and DV69, $PVY^{N\text{-}Wilga}$: 10766 and finally PVY^{O}: DV71. The primers used for full length sequencing are presented in Table 2.1. These were analysed against isolates detailed in Table 2.2. The evolutionary history was inferred using the Neighbor-Joining method and evolutionary distances were computed using the number of differences method (Nei and Kumar, 2000) and are in the units of the number

of amino acid differences per sequence. The coding data was translated assuming a Standard genetic code table. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

2.3. Results

2.3.1 Serological survey of PVY field samples.

In total 1,454 leaf samples submitted for testing by ELISA as being symptomatic for virus symptoms during the 2010 growing crop inspections. These were all tested for PVY using both monoclonal and polyclonal antibodies (SASA Virology laboratory report 2010) to ensure that serological testing is accurately detecting all symptomatic PVY infected plants in the field. In total 911 samples tested positive for PVY with SASA PAb PVY from which 819 PVY isolates were positives for SASA MAb PVY^{N} and 92 isolates were positive for SASA MAb $PVY^{O/C}$. The conclusions of this survey revealed no differences in the DAS-ELISA results using monoclonal $PVY^{O/C}$ and PVY^{N} antibodies and PVY Polyclonal antibodies. There were no symptomatic leaf samples that reacted with the PVY PAb without also reacting to a PVY MAb. This suggest that in spite of the likely molecular diversity of PVY field isolates, all of the PVY isolates present in Scotland are currently be detected using the monoclonal antibodies SASA MAb PVY^{N} and SASA MAb $PVY^{O/C}$.

2.3.2. Sequencing of PVY genomic recombinant junctions R1, R2 and R3.

Recombinant junctions (RJs) between PVY^O and PVY^N genomes have been identified in PVY (Lorenzen *et al.,* 2006) with differences between PVY isolates serotypes, biotypes and molecular types (variants), at various positions within the genome: approximately nucleotide (nt) 500, nt 2300, nt 5715 and also in several areas between 7940 and 9460bp along the genome (Schubert *et al.,* 2007). Genomic segments spanning these RJs harbour a high genetic diversity due to the more frequent occurrence of recombination breakpoints, as opposed to other more conserved region of the genome. Therefore isolates were sequenced over these recombinant junctions to determine if sequencing across a recombinant junction can be used as an alternative to full length sequencing and if so which junction or junctions gives the most accurate discrimination.

- R1= 1010bp in length and covers from 2035bp to 3043bp which includes the second part of the coding region for the HC-Pro into the P3.
- R2 = 1396bp in length and covers from 5277bp to 6673bp which includes part of the CI protein all of the 6K2 and VPg, and part of the NIa
- R3=1568bp in length and covers from 7749-9594, which includes part of the NIb and the full Coat Protein coding regions.

Sections 2.3.2.1, 2.3.2.2 and 2.3.2.3 will detail the results from the sequencing of each of these Recombinant Junctions (RJ). In addition the individual protein components which make up each RJ has been sequenced separately for comparison and in order to identify areas of the genome with high variation.

2.3.2.1. Phylogeny of PVY genomic recombinant junction (RJ) R1

Phylogenetic analysis of the R1 RJ separate PVY isolates into 5 clades. Of the 60 isolates which generated exploitable sequence data for this region, the distribution of PVY isolates is as follow: 46 EU-NTN, 4 N and 7 NA. The three PVY^{O} isolates genome retrieved from GenBank form a separate cluster. Contained within the EU-NTN clade, isolates identified biologically as N-Wilga form a separate subgroup. However, further analysis using additional N-Wilga isolates would be required to confirm if most/all N-Wilga isolates can be similarly clustered. There are further subgroupings within the EU-NTN clade, which might suggest further molecular diversity within the main EU-NTN groupings. Overall, all tested molecular PVY variants have been accurately classified by sequencing across the R1 genome.

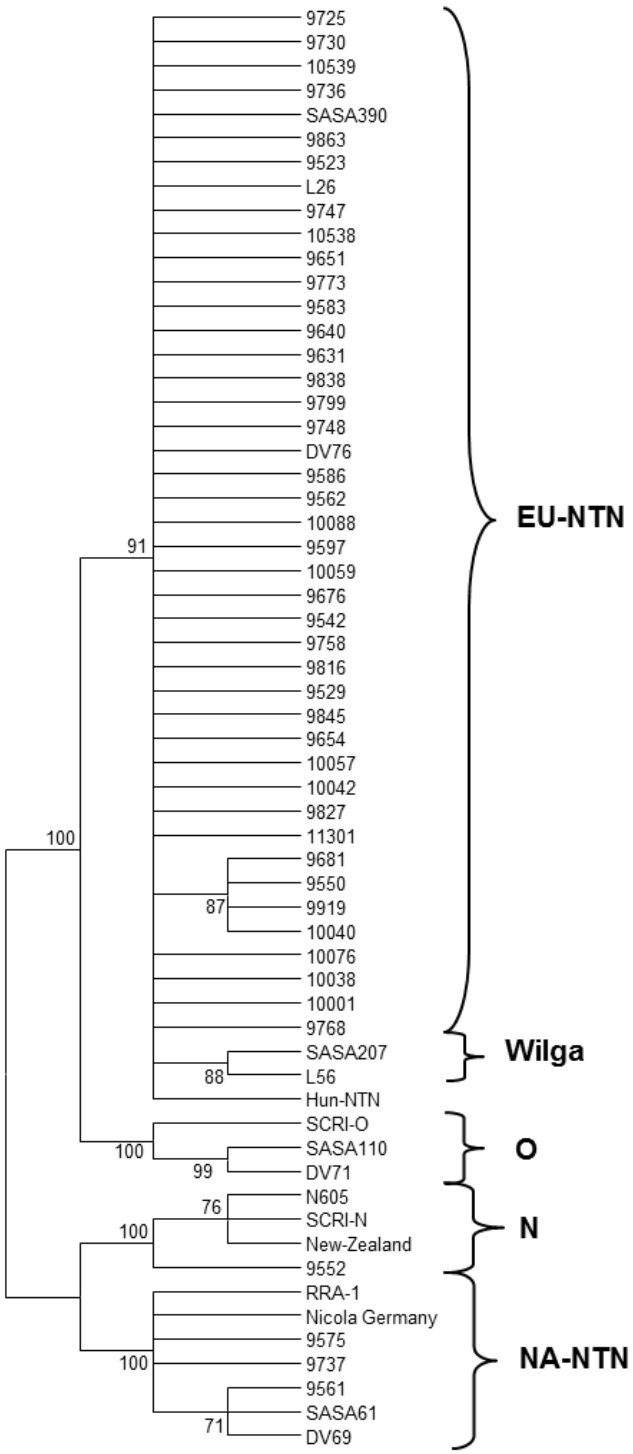


Figure 2.3 Evolutionary relationship of PVY isolates for the R1 region of the genome. In total 60 isolates were analysed for this region. The evolutionary distances were computed using the Tamura-Nei (1993) method and bootstrapped values of less than 70 were collapsed. In total after editing of the sequences 706 nucleotide positions were included in the final dataset.

2.3.2.1.1. The HC-Pro region of R1

The HC-Pro component of the R1 region does not display the level of molecular diversity as found for the complete R1 region. Of the 98 isolates sequenced, 82 fall within the unified EU-NTN/N/Wilga clade, while 11 clustered within the NA-NTN clade and the 3 PVY^{O} isolate form a separate clade, although this clade can now be resolved in two separate sub-groups. It is not possible to separate N, EU-NTN and Wilga isolates, suggesting that, as previously reported, a similar nucleic acid composition for HC-Pro between these variants. Contrastingly, all NA-NTN isolates remained clustered which confirms their divergent nucleic acid composition.

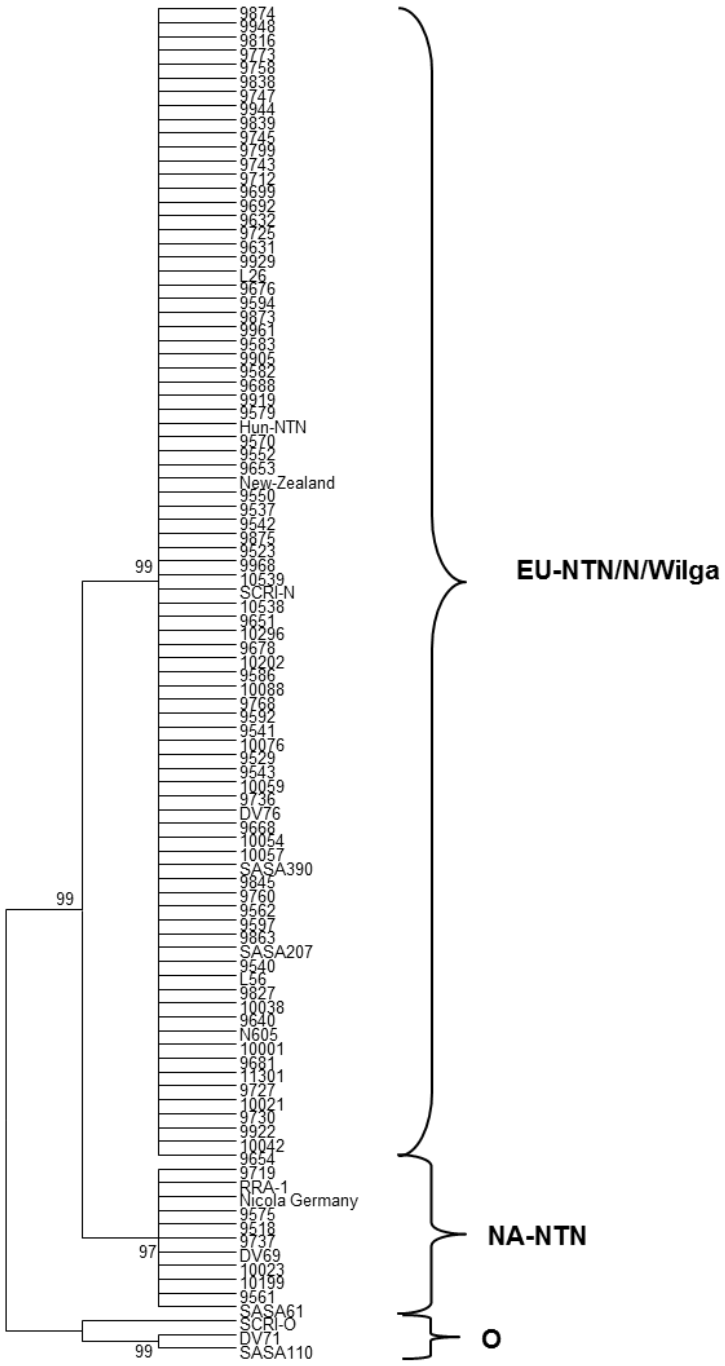


Figure 2.4 Evolutionary relationship of PVY isolates for the HC-Pro component of the R1 region of the genome. In total 98 isolates were successfully analysed for this region. The evolutionary distances were computed using the Tamura-Nei (1993). There were a total of 246 nucleotide positions included the final dataset.

2.3.2.1.2. The P3 region of R1

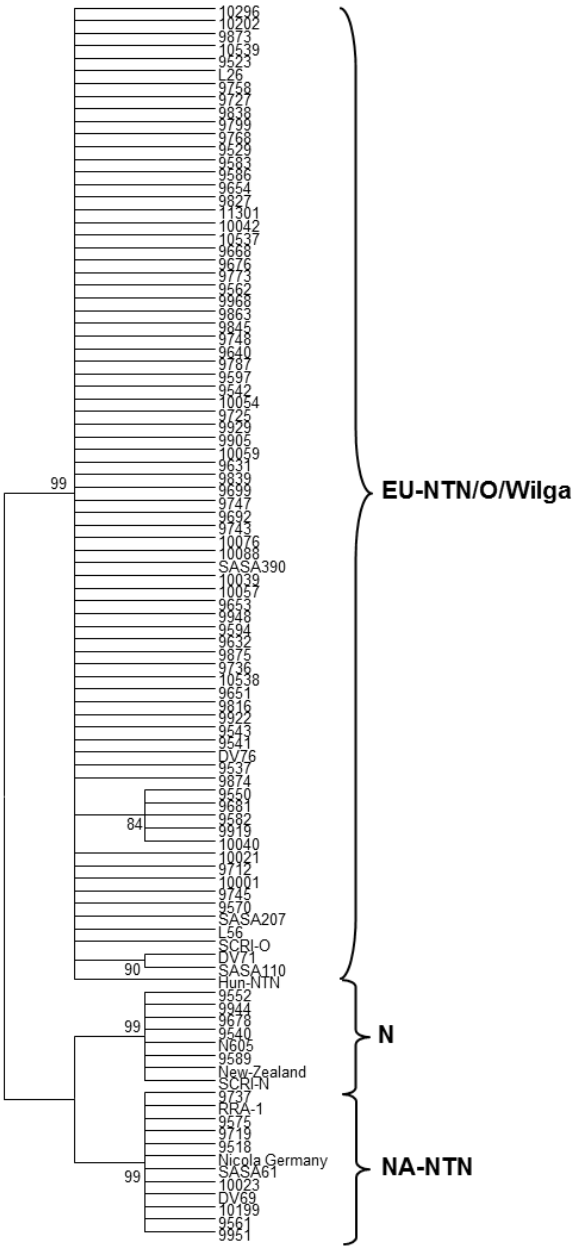


Figure 2.5 Evolutionary relationship of PVY isolates for the P3 component of the R1 region of the genome. In total 98 isolates were successfully analysed for this region. The evolutionary distances were computed using the Tamura-Nei (1993) method. There were a total of 339 nucleotide positions recorded for this dataset.

The P3 region of the R1 RJ, display an even lower degree of diversification than the full R1 region. P3 sequencing defines three main clades for the 76 PVY isolates sequenced which now group EU-NTN/Wilga/O in a single clade, and 2 separate clades N (8 isolates) and NA-NTN (12 isolates). Two of the PVY^{O} isolates are separated out within the EU-NTN clade, while the other clustered within the main EU-NTN group.

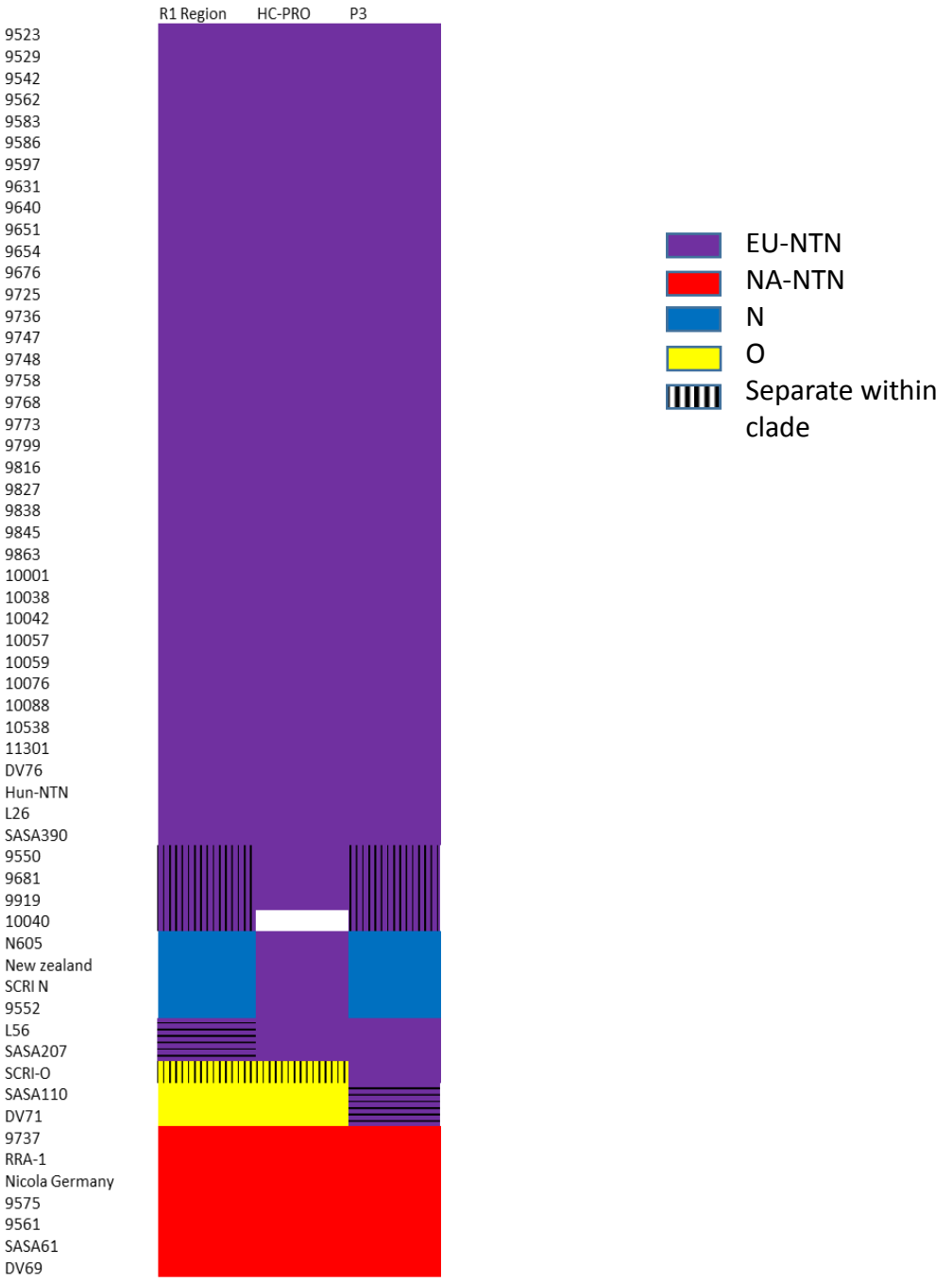


Figure 2.6. A schematic representation of the R1 region. Column one is a schematic of the full R1 phylogenetic tree, column 2 is a schematic of the HC-Pro phylogenetic tree and column 3 is a schematic of the P3 phylogenetic tree.

Over the complete R1 RJ region, a maximum of 14 amino acid differences were found between two different isolates with a maximum of 117 nucleotide changes between two different isolates. This shows that many of the nucleotide changes are synonomous and do not therefore affect the amino acid composition of the proteins. This suggests that these changes are under strong selective pressure to maintain an identical protein composition to fulfil its function. It was also possible to distinguish different strains using synonymous changes only. The schematic figure 2.6 demonstrates that sequencing of the R1 RJ region enables us to classify PVY isolates more accurately than sequencing only either P3 component or HC-Pro, suggesting that R1 RJ is a suitable region for targeted sequencing and molecular typing of PVY isolates. The N-Wilga was clustered in a separate group within the EU-NTN clade and although not fully separated from the EU-NTN, if used in conjunction with an initial serological diagnosis as PVY^{O} serotype the R1 region can be used to confirm its classification as Wilga without the need for a bioassay or full length sequencing.

2.3.2.2. Phylogeny of the R2 recombinant junction

As with the R1 RJ, the R2 RJ region accurately defined the PVY isolates into all previously identified clades, 30 of the 48 PVY isolates sequenced in the R2 RJ clustered within the EU-NTN clade, 5 within the NA-NTN and 6 within the N groups. SCRI-O clustered with the Wilga isolates, suggesting that this region of SCRI-O is more closely related to Wilga isolates, while the remainder PVY^{O} isolates clustered together to form a separate clade

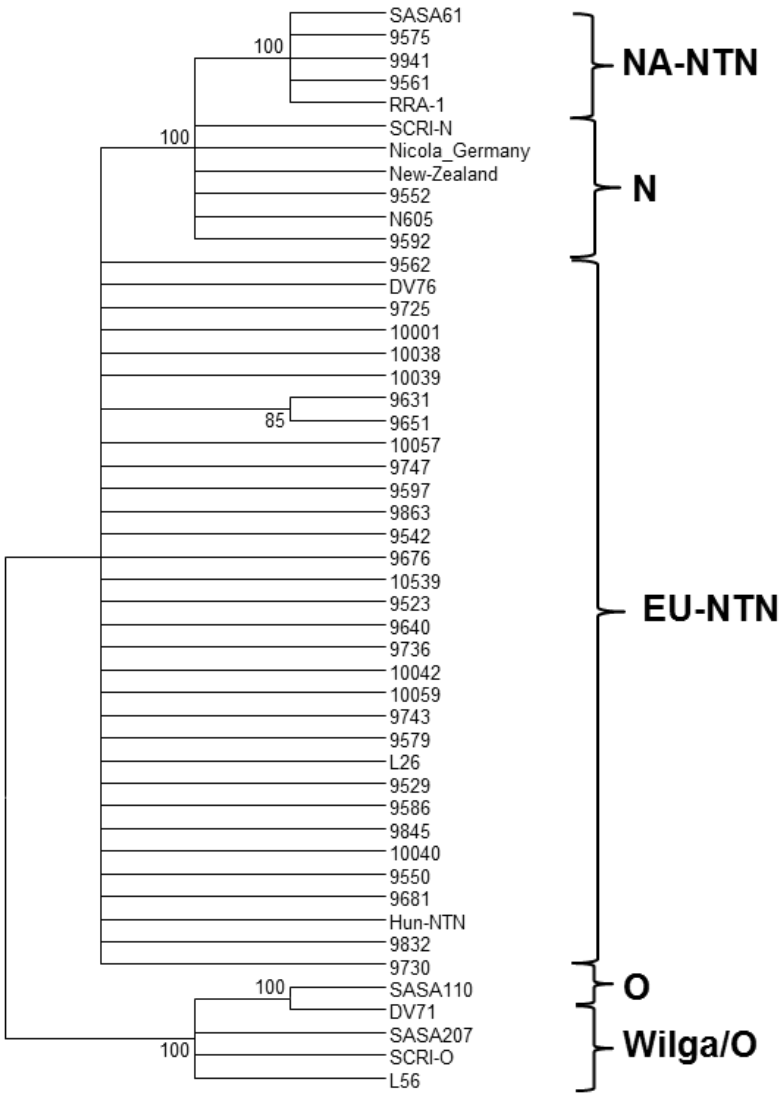


Figure 2.7 Evolutionary relationship of PVY isolates for the R2 region of the genome. In total 48 isolates were successfully analysed for this region. The evolutionary distances were computed using the Tamura-Nei (1993) method and are in the units of the number of base substitutions per site. There were 1031 nucleotide positions in the final dataset.

2.3.2.2.1 The CI region of the R2 recombinant junction

The CI region only separates isolates in to two clade, with 48 isolates within the EU-NTN group and the remainder 13 isolates within the NA-NTN/N group.

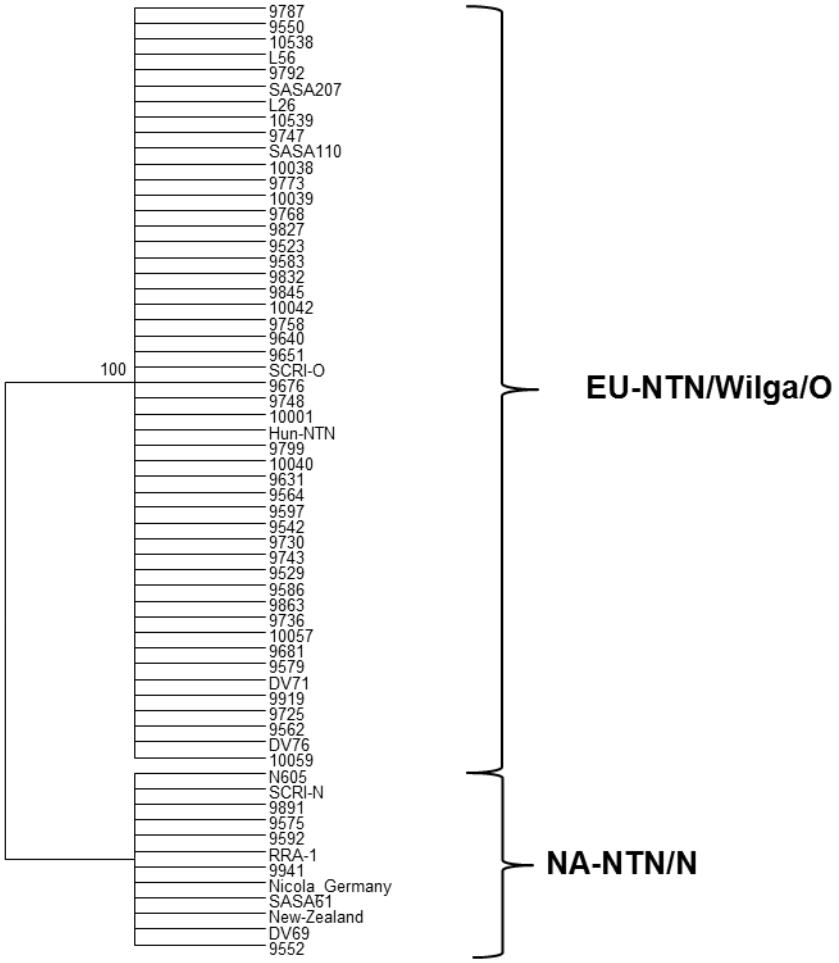


Figure 2.8 Evolutionary relationship of PVY isolates for the R1 region of the genome. In total 61 isolates were successfully analysed for this region. The evolutionary distances were computed using the Tamura-Nei (1993) method and are in the units of the number of base substitutions per site. There were 85 nucleotide positions in the final dataset.

2.3.2.2.2 The 6K2 region of the R2 recombinant junction

The entire 6K2 protein was sequenced. The phylogenetic tree of 6K2 generated 3 clades, where the O and Wilga groups fall within the EU-NTN clade which contains 35 isolates. 7 and 6 isolates fall within the NA-NTN and N groups respectively.

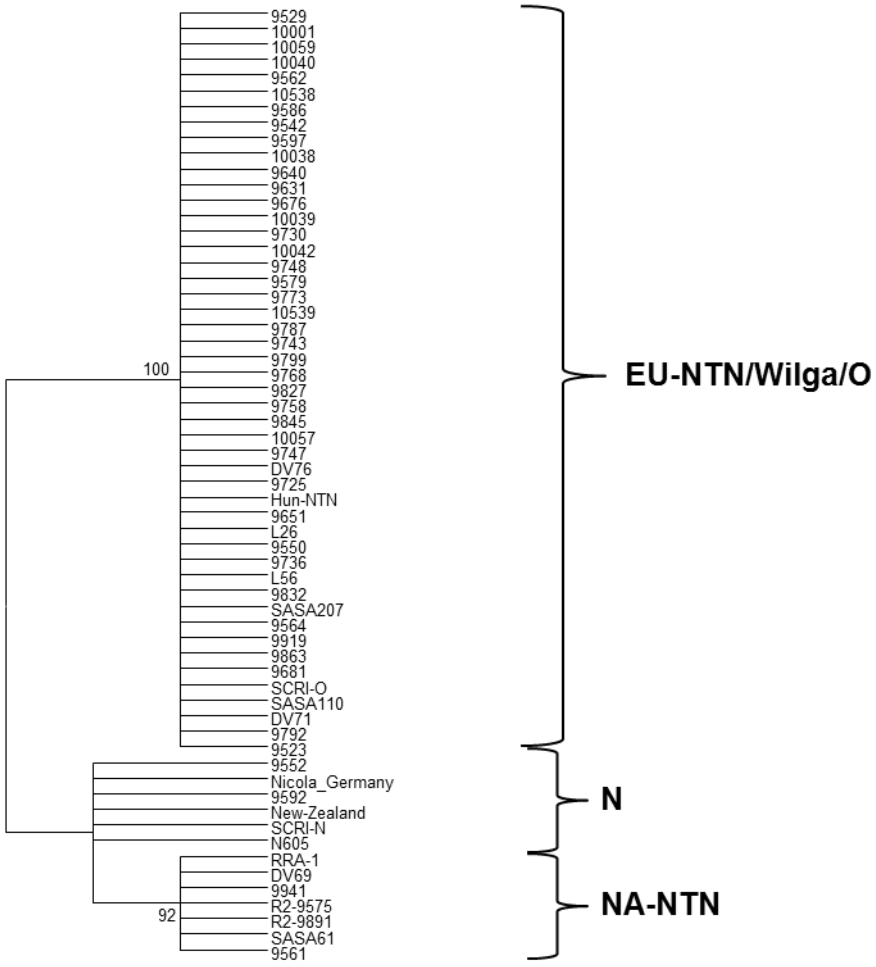


Figure 2.9 Evolutionary relationship of PVY isolates for the 6K2 protein within the R2 region of the genome. In total 61 isolates were successfully analysed for this region. The evolutionary distances were computed using the Tamura-Nei (1993) method and are in the units of the number of base substitutions per site. There were 153 nucleotide positions in the final dataset.

2.3.2.2.3 The VPg region of the R2 recombinant junction

The VPg displays the highest level of variability within the R2 region. The NA-NTN group is separated into 3 subgroups, and the EU-NTN group is separated into two subgroup, of which one minor subgroup is defined by 2 isolates. The N clade form a single group, while O and Wilga families are separated with SCRI-O now clustering within the Wilga clade.

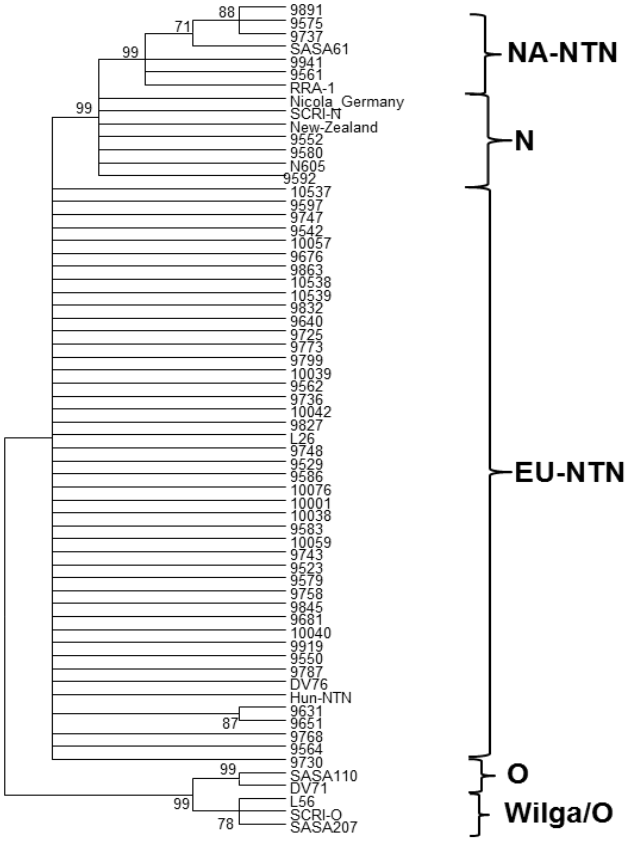


Figure 2.10 Evolutionary relationship of PVY isolates for the VPg protein within the R2 region of the genome. In total 64 isolates were successfully analysed for this region. The evolutionary distances were computed using the Tamura-Nei (1993) method and are in the units of the number of base substitutions per site. There were 552 nucleotide positions in the final dataset.

2.3.2.2.4 The NIa region of the R2 recombinant junction

The NIa phylogeny define 3 separate clades. EU-NTN, N and NA-NTN are all clustered in one single group for the 46 isolates sequenced. Wilga and O isolates are separated in different clades, and further nucleotide diversity in the NIa region was found between the two Wilga isolates SASA207 and L56 that can be separated from each other.

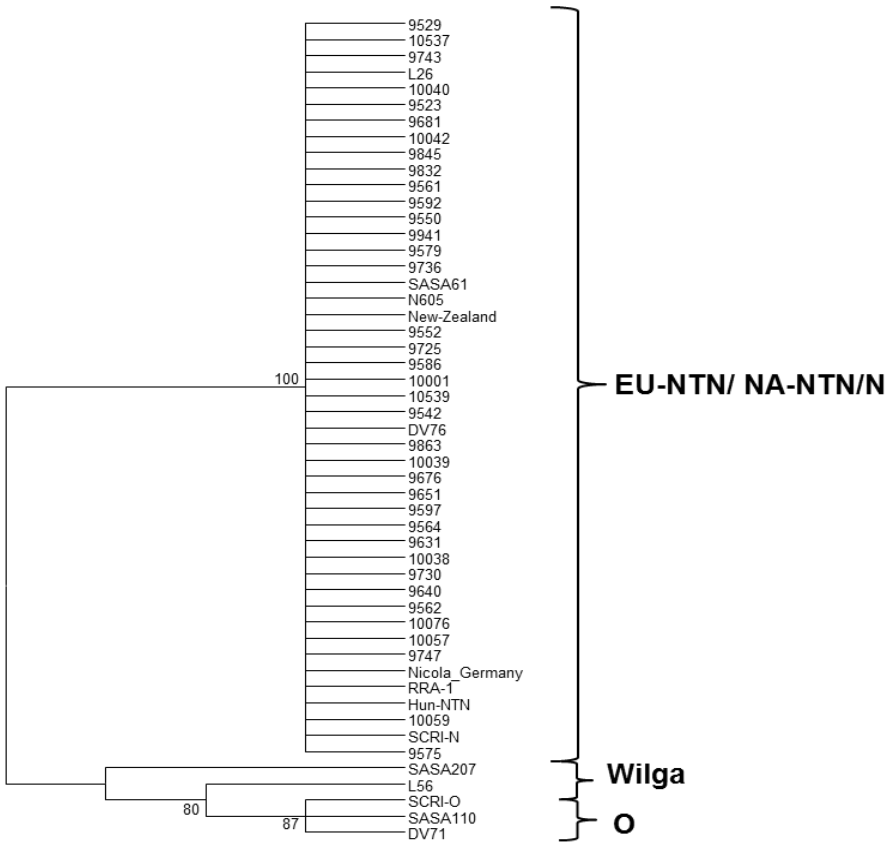


Figure 2.11 Evolutionary relationship of PVY isolates for the NIa component of the R2 region of the genome. In total 51 isolates were successfully analysed for this region. The evolutionary distances were computed using the Tamura-Nei (1993) method and are in the units of the number of base substitutions per site. There were 210 nucleotide positions in the final dataset.

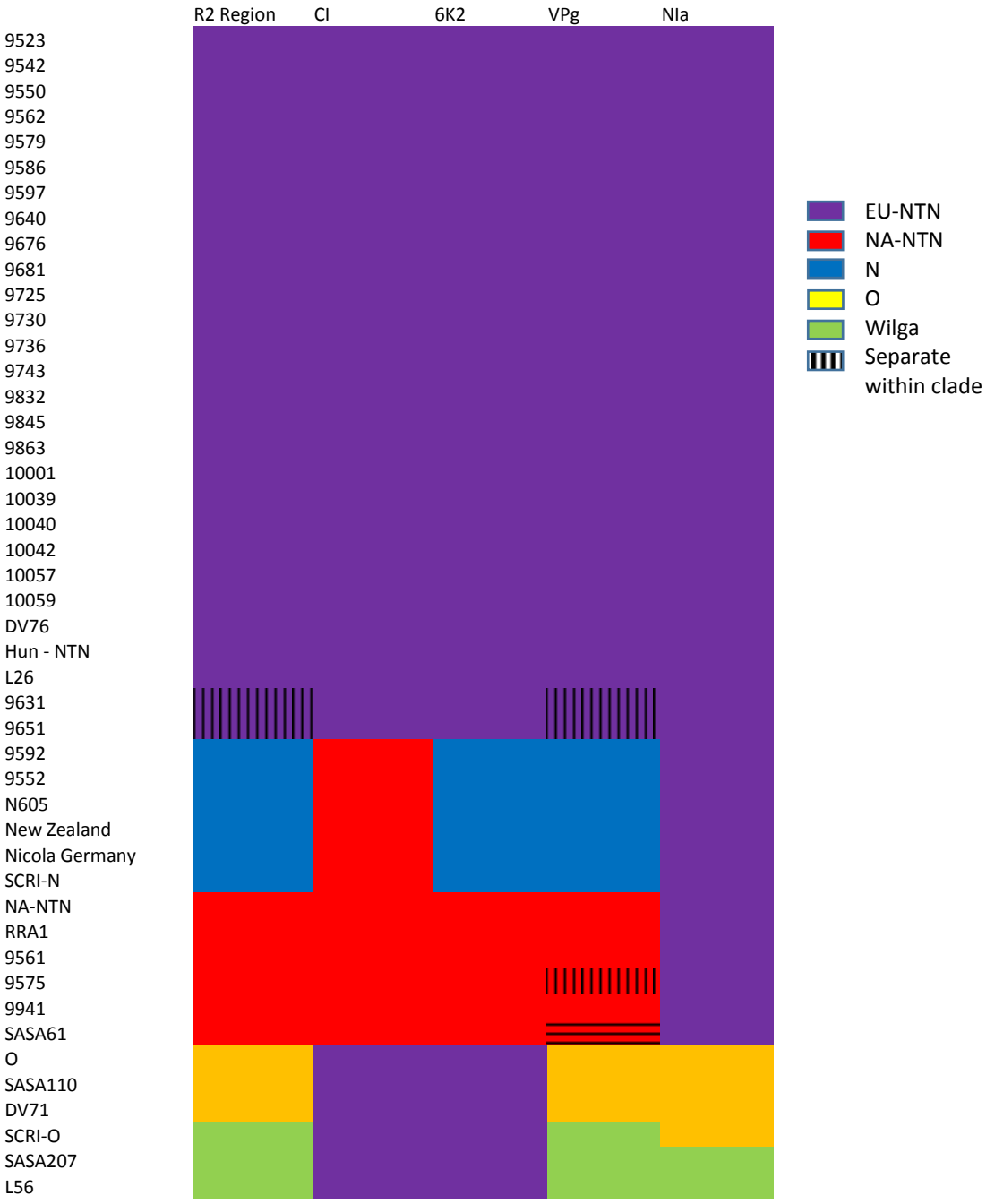


Figure 2.12 Schematic representation of the R2 region. Column one is a schematic of the full R2 phylogenetic tree, columns 2 to 5 are schematic representations of the phylogenetic trees of the individual protein sequences within the R2 region.

Overall the phylogenetic analysis of the complete R2 RJ region gives a good representation of the molecular diversity of PVY isolates by accurately defining previously identified clades. One notable exception being that SCRI-O that groups with the Wilga isolates, this isolate only clusters with the other PVY^{O} isolates in the NIa protein. There was a higher degree of nucleotide diversity of PVY isolates within the NA-NTN group, whereby 3 different subgroups were defined. A total of 29 amino acid differences and 182 nucleotide substitutions between the most divergent isolates was identified within the R2 region.

2.3.2.3 Phylogeny of the R3 recominant junction

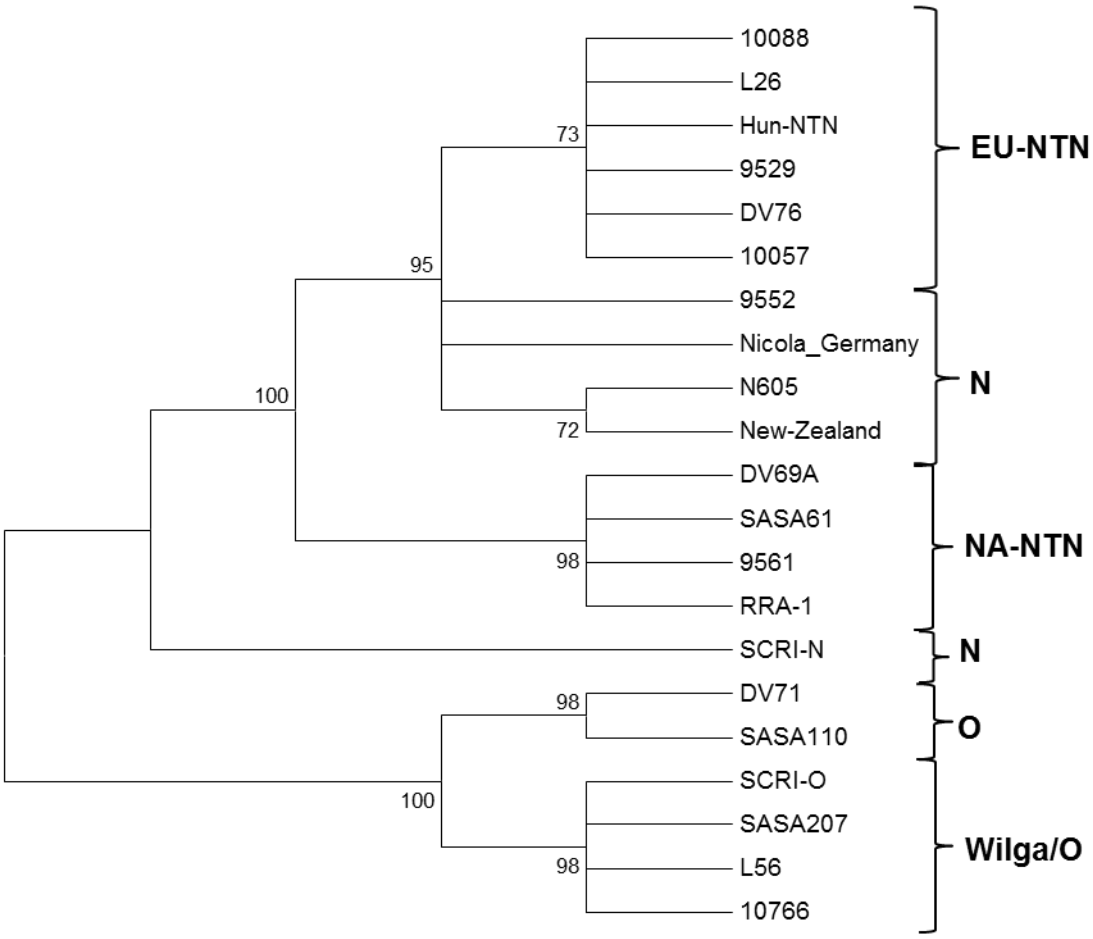


Figure 2.13 Evolutionary relationship of PVY isolates for the R3 region of the genome. In total 21 isolates were successfully analysed for this region. The evolutionary distances were computed using the Tamura-Nei (1993) method and are in the units of the number of base substitutions per site. There were 1001 positions in the final dataset.

The R3 RJ region encompasses both NIb and CP proteins. Full sequencing of the R3 region accurately separates all isolates into the previously defined molecular clades, with the exception of SCRI-O which is now clustered with

the Wilga isolate L56 and SASA207. The SCRI-N isolate forms a separate subgroup from the other N types (N605, New Zealand, Nicola-Germany), which appear more closely related to EU-NTN isolates.

2.3.2.3.1 The NIb region of the R3 recombinant junction

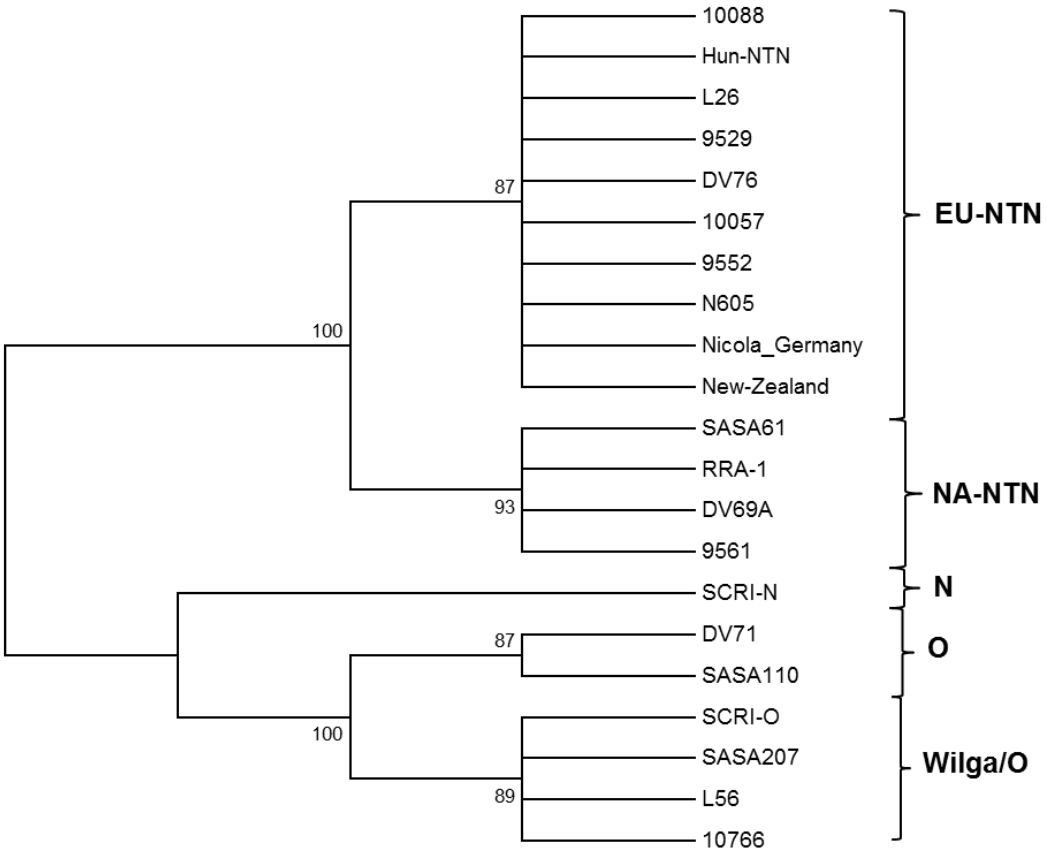


Figure 2.14 Evolutionary relationship of PVY isolates for the NIb component of the R3 region of the genome. In total 21 isolates were successfully analysed for this region. The evolutionary distances were computed using the Tamura-Nei (1993) method and are in the units of the number of base substitutions per site. There were 663 positions in the final dataset.

The NIb phylogeny clustered isolates N605 and New Zealand of the N-group within the EU-NTN clade, with the exception of SCRI-N that defines a separate group more closely related to O and Wilga isolates than other N types. SCRI-O clusters within the Wilga clade.

2.3.2.3.2. Phylogenetic analysis of the CP

The CP nucleotide sequence is often used to discriminate PVY isolates at the molecular level. CP sequencing appears to discriminate all previously identified molecular groups. Again SCRI-O clusters with the Wilga isolates. Overall 62 isolates fall within the EU-NTN clade, 10 N, 11 NA, 3 O and 4 O/Wilga.

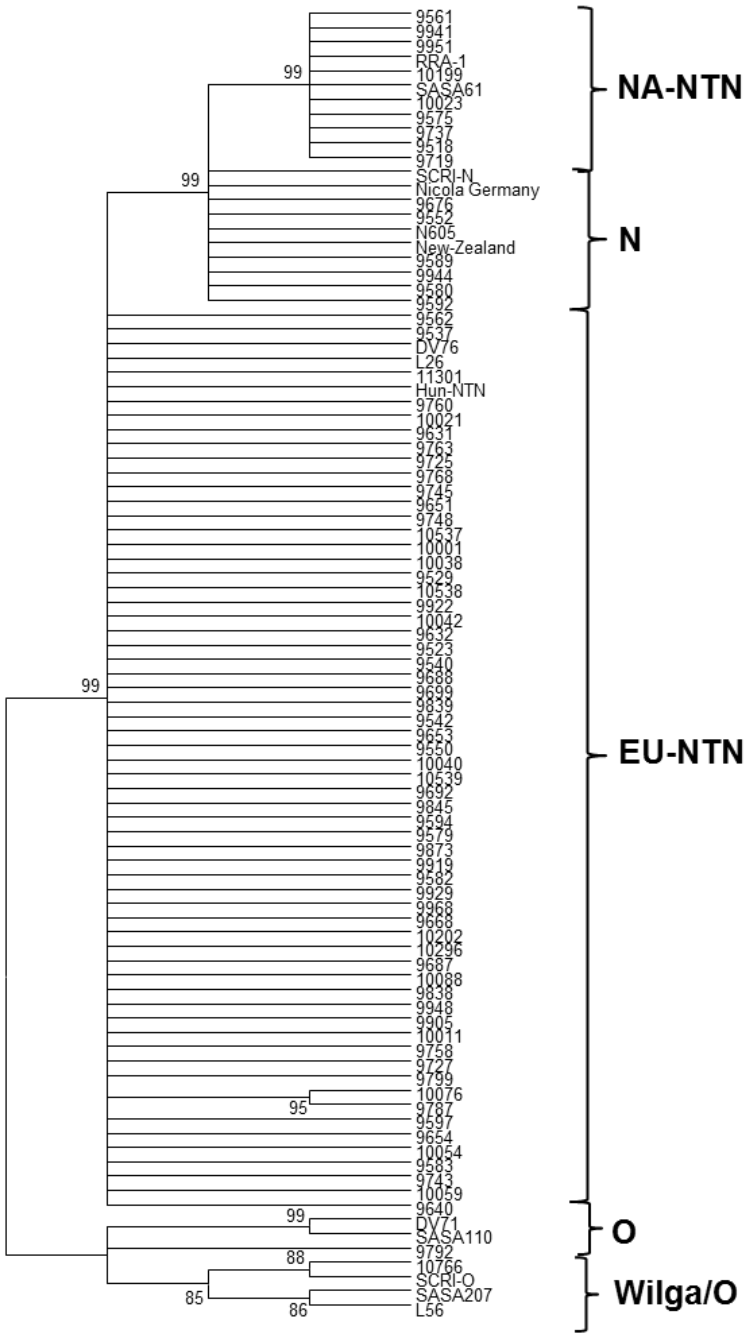


Figure 2.15 Evolutionary relationship of PVY isolates for the CP component of the R3 region of the genome. In total 91 isolates were successfully analysed for this region. The evolutionary distances were computed using the Tamura-Nei (1993) method and are in the units of the number of base substitutions per site. There were 804 nucleotide positions in the final dataset as this included the full coding region for the CP.

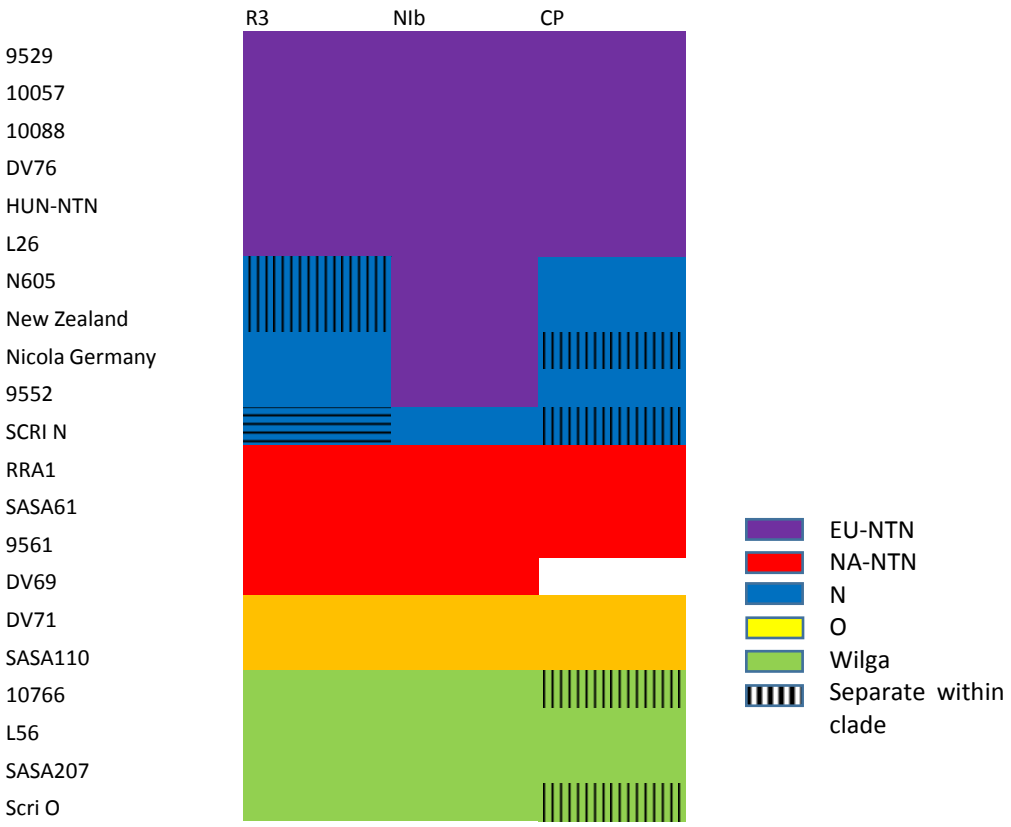


Figure 2.16 Schematic representation of the R3 region. Column one is a schematic of the full R3 phylogenetic tree, column 2 is a schematic of the NIb phylogenetic tree and column 3 is a schematic of the Coat Protein phylogenetic tree.

The analysis of the full R3 region was limited to 21 PVY isolates. Overall 31 amino acid differences are recorded for this region and 207 nucleotide differences.

2.3.2.4 Summary of the phylogenetic analysis of R1-R2-R3 regions

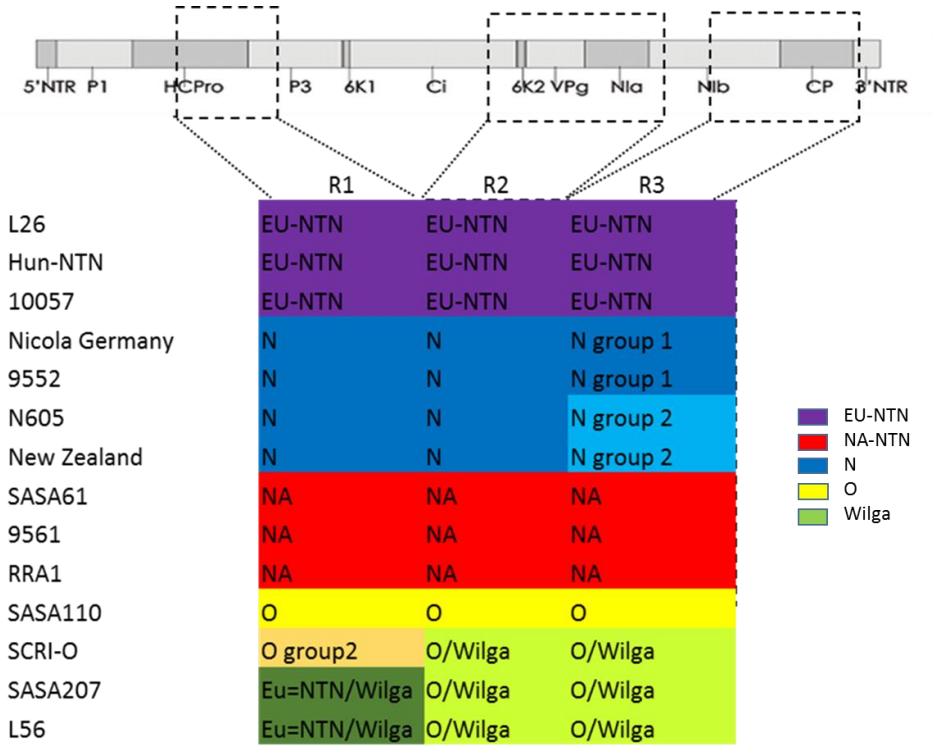


Figure 2.17 Schematic summary of classification of isolates within the R1, R2 and R3 recombinant junction regions.

Figure 2.17 above is a summary of the R1, R2 and R3 regions using previously characterized PVY isolates worldwide along with representative Scottish field isolates. It demonstrates that targeted sequencing of recombinant junctions is a valid method to discriminate and group PVY isolates in their respective molecular clades. Our study highlighted notable differences between O and Wilga isolates confirming, as previously reported, that their genome derive from multiple recombination events between O and N parent isolates. Our result suggest that-partial sequencing of PVY genome targeted to the R1 RJ is a suitable approach to discriminate PVY molecular variants.

Overall the phylogenetic analysis of *ca* 100 PVY^{N} field isolates revealed that most of the isolates, regardless of the variety or geographical location they originate from, show a high level of similarity, with 70-80% of isolates clustering with previously identified EU-NTN isolates. This would suggest that using molecular characterisation of isolates, the majority of those identified in the field in Scotland are relatively homogenous and lie predominantly within the $PVY^{EU\text{-}NTN}$. There appeared to be less variation within the EU-NTN as a higher level of molecular diversity was found for isolates of the NA-NTN or Wilga groups where individual isolates clustered within additional molecular subgroups or shifted between different clades along the genome. The reason for this diversity might be due to selective pressure that could in limited circumstances advantage a variety of isolates over the well-established prevalent isolates which are likely to be generally fitter and over-represented in field populations.

2.3.3 Full length sequencing of selected PVY isolates

Full length sequencing was undertaken for 10 isolates, selected on the basis of their previously defined molecular groupings (*i.e.* R1, R2 and R3 RJ sequencing) and also their biological features (the study of PVY pathogenicity is addressed in Chapter 3). Whole genome analysis confirmed previous analysis using R1 partial sequencing in grouping in a similar way the selected PVY isolates in their respective molecular groups:

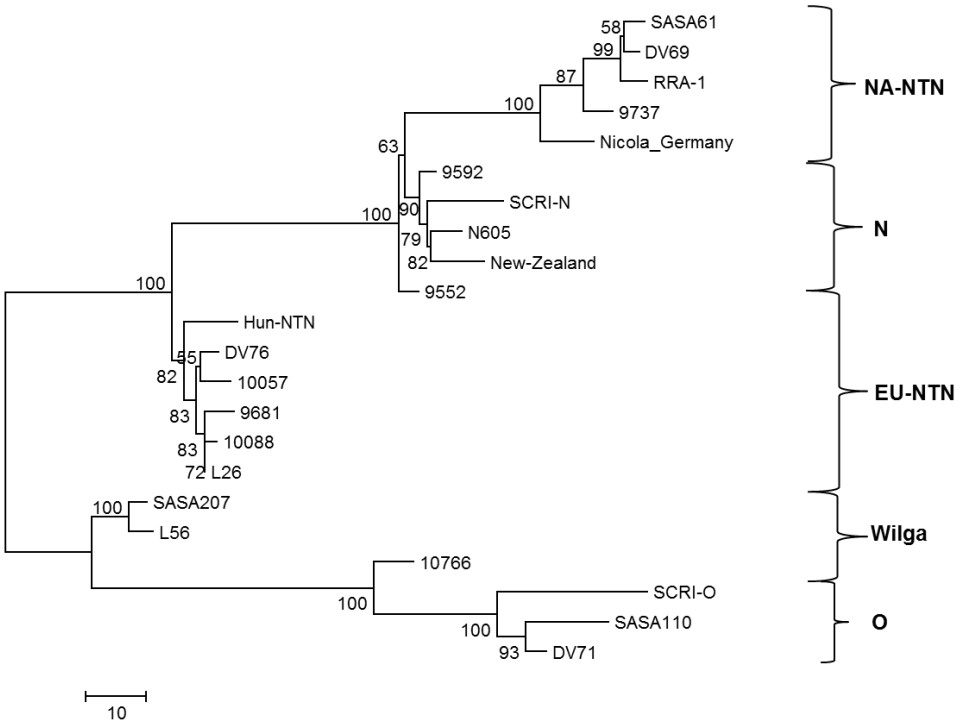
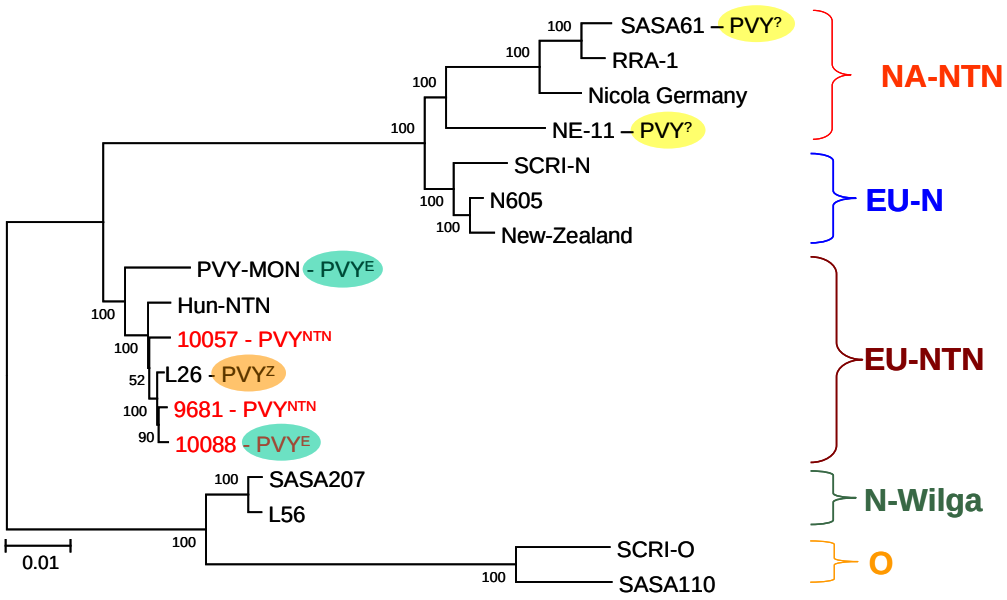
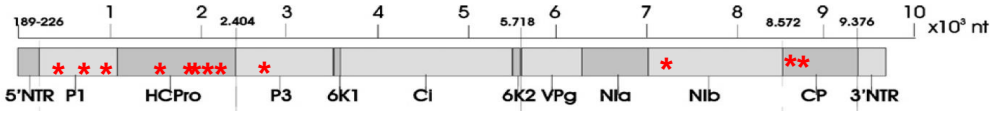


Figure 2.18 Full length sequencing of a selection of Scottish isolates. This phylogenetic tree was constructed using the neighbour joining method on MEGA 5 for number of differences in amino acids. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches.

Further analysis was undertaken with the view to link the molecular groups and the biological properties of the selected isolates. Of particular interest, PVY isolate 10088 does not trigger vein necrosis in tobacco despite being serologically identified as PVY^{N} (Figure 2.19). Further indexing on potatoes harbouring *Nc*, *Ny* and *Nz* resistance genes identified isolate 10088 as a PVY^{E} strain (see Chapter 3 section 3.3.3). Phylogenetic analysis of the whole genome revealed that isolates 10088, 9681 and 10057 are closely related at the molecular level, although both 9681 and 10057 elicit vein necrosis in tobacco.





	Strain biotype	PVY^{Z}	PVY^{E}	PVY^{E}	PVY^{N}	PVY$^{E\ or\ Z}$	PVY^{N}	PVY$^{E\ or\ Z}$	PVY^{O}
	Symptoms	M	M	M	VN	M	VN	M	M
	Molecular type	EU-NTN	EU-NTN	EU/NA-NTN	EU-NTN	NA-NTN	NA-NTN	NA-NTN	O
	Isolate	L26	10088	PVY-MON	9681	NE-11	RRA-1	SASA61	SASA110
P1	nt 63	S	S	S	F	S	S	S	L
	nt 196	M	M	M	I	M	M	M	M
	nt 225	T	T	T	A	T	T	T	S
HC-Pro	nt 205	G	D	D	D	D	D	D	D
	nt 339	N	N	N	N	N	N	N	D
	nt 340	S	T	S	S	S	S	S	S
	nt 400	K	K	K	K	K	K	K	R
	nt 419	E	E	E	E	E	E	E	D
P3	nt 120	K	K	K	R	K	K	K	K
NIb	nt 606	S	S	S	N	S	S	S	G
CP	nt 6	N	I	N	N	N	N	N	N
	nt 33	T	T	N	T	N	T	T	S

Figure 2.19: Whole genome phylogeny of selected PVY field isolates and molecular comparison with other PVY isolates with known biological properties. Upper panel: Whole genome phylogenetic analysis of selected PVY field isolates. Molecular groups are indicated on the right. Isolates eliciting vein necrosis in tobaccos are: SASA207 (N-Wilga), 9681 and 10057 (EU-NTN), SCRI-N, N605, New Zealand (EU-N), RRA1 (NA-NTN). Isolates eliciting mosaic on tobaccos are: SCRI-O and SASA110 (O), 10088, L26 and MON (EU-NTN), SASA61 and NE11 (NA-NTN). Central Panel: Schematic

representation of nucleotide diversity along the PVY genome thought to be associated with vein necrotizing ability (indicated by *). Lower Panel: Table summarizing the biological, serological and molecular groups of selected PVY isolates and the amino acid changes thought to be associated with vein necrotising abilities in tobacco. The corresponding PVY protein is indicated on the left.

The mapping of genetic determinants of vein necrosis in tobacco is summarized in Figure 2.19. From the amino acid composition of the isolate studied here and of others previously published, there is currently no apparent consensus on a single shared amino acid associated with Mosaic (M) or Vein Necrosis (VN) elicitation in all these isolates. Our study suggests that biological groups of PVY do not cluster in the same molecular group.

Amino acid differences between isolates from full length sequencing, of the Scottish isolates included are 10057 and 10088 as these were successfully sequenced with no gaps across the genome. 10057 is an EU-NTN and 10088 molecularly clusters with known $PVY^{EU\text{-}NTN}$ isolates, even though it is biologically an E type. The lowest level of variation was between 10088 and L26 which had only 4 differences across the genome, both of these isolates are PVY^{E}. The highest variation was between SCRI-O and SASA 61 with 309 differences across the genome suggesting a high level of divergence for the NA-NTN compared to the O types.

Table 2.3 Number of amino acid differences per full length sequence

The analysis involved amino acid sequences of 14 PVY isolates. The coding data was translated assuming a Standard genetic code table. All positions containing gaps and missing data were eliminated. There were a total of 2975 amino acid positions in the final dataset. Evolutionary analyses were conducted in ME

	SASA61	RRA-1	Nicola_Germany	N605	New-Zealand	SCRI-N	Hun-NTN	10057	10088	L26	SASA207	L56	SCRI-O	SASA110
SASA61														
RRA-1	16													
Nicola_Germany	34	29												
N605	69	64	57											
New-Zealand	76	71	68	21										
SCRI-N	79	74	71	24	31									
Hun-NTN	138	131	125	87	93	95								
10057	129	124	119	82	89	92	24							
10088	129	124	117	79	86	89	21	11						
L26	128	123	116	77	84	87	19	9	4					
SASA207	190	185	180	142	147	141	85	77	76	74				
L56	191	186	181	143	148	142	86	78	77	75	9			
SCRI-O	309	305	300	299	304	298	243	239	238	237	175	175		
SASA110	289	285	280	281	286	278	225	221	220	219	165	165	78	

2.4 Discussion

2.4.1. Limitations of serological testing methods

Monoclonal antibodies are widely used for routine diagnostics in testing laboratories worldwide for many years. DAS_ELISA testing is an essential method for seed potato certification, being cost-effective and high-throughput, which over the years has proven to be a reliable method to identify so far all PVY Scottish field isolates into two main serotypes PVY^{N} and $PVY^{O/C}$. Recombination events in the evolution of PVY with PVY^{O} and PVY^{N} have made the differentiation of strains using serological methods more challenging. An example of this is the recombinant $PVY^{N\text{-}Wilga}$ strain which is recognised as PVY^{O} serologically despite inducing vein necrosis in tobacco cv Xanthi and White Burley. Authors have also recently described incidences of strain misidentification using monoclonal antibodies (Karasev *et al.*, 2010).

Galvino-Costa *et al* (2012) reported that two Brazilian recombinant isolates of PVY (a PVY^{NTN} and the PVY^{E} strain group) were not detected using SASA PVY^{N} antibodies despite them having a similar genome to PVY^{N} types. The authors did not use in conjunction SASA $PVY^{O/C}$ Mab and there is still the possibility that these isolates might harbour epitopes that could be recognized by SASA $PVY^{O/C}$ Mab. For this reason, it was important to establish if all isolates present in the field in Scotland are readily detected using SASA monoclonal antibodies. The epitopes for SASA monoclonal antibodies are located within the coat protein (CP) coding region that displays high levels of

variation between isolates. In the past few years, the occurrence of isolates that have been misidentified by ELISA with some monoclonal antibodies has been increasing, suggesting that changes in the CP can lead to a loss of cross-reactivity (Nikolaeva *et al.*, 2012). This issue can be addressed by using polyclonal antibodies which bind to multiple CP epitopes. The survey of all samples submitted to the laboratory in 2010 using both monoclonal and polyclonal antibodies indicated that all Scottish isolates of PVY are currently detected using monoclonal antibodies. However, to ensure all PVY isolates are being detected in Scotland, regular surveys using PVY polyclonal antibodies is advisable.

2.4.2. Molecular methods used for the detection of PVY

Molecular techniques enable the characterisation of PVY isolates on the basis of their genomic sequence. Genome sequencing makes it possible to distinguish between different strains, and variants within serological or molecular groups. However, full length sequencing of PVY is expensive and time consuming. For this reason other molecular methods of classification of isolates have been used.

Reverse Transcription PCR methods have been designed to detect small levels of virus within plant cells. This technology is more sensitive than ELISA at detecting low levels of virus present both within a growing plant or a dormant

tuber. RT-PCR can be used either in simplex where the presence just one virus or virus strain is monitored, or multiplex where several viruses or virus strains can be detected simultaneously. A limitation of traditional RT-PCR is that they require to be run on a gel electrophoresis.

Real-Time RT-PCR allows the rapid detection of one or more strains of the virus within a single well. It can even distinguish between isolates on the basis of point mutations. Real-Time RT-PCR methods have been reported to discriminate between PVY^{N} and PVY^{O} (Balme-Sinibaldi *et al*, 2006) also PVY^{N} and PVY^{NTN}, however, due to the very high molecular diversity of PVY isolates validation of these primers and probes is challenging and require frequent re-assessment of primers used to distinguish between different strains of the virus. (Chikh Ali *et al*., 2009).

Single Nucleotide Polymorphism (SNP) have been identified on the PVY genome, which are believed to be associated with the biological properties of PVY isolates. These include a range of methods (including SNaPshot assay). However none of these are adapted for high-throughput typing (Rolland *et al.*, 2008).

2.4.3 Assessment of the use of R1, R2, R3 regions for molecular typing

The recombinant junctions R1, R2 and R3 are likely to cover the main areas of recombination across the PVY genome and for this reason we hypothesised that these segments of PVY genome would be the most informative with

respect to the molecular nature of the isolates without the need for full-length sequencing. All three regions tested gave accurate distinction between the different PVY virus strains with the exception of $PVY^{N\text{-}Wilga}$. However, this issue could be overcome by either analysing more than one region including R1, where Wilga is grouped with the EU-NTN and with the O types for the other regions. Alternatively if an isolate has been serotyped as N or O/C in a first instance, sequencing the R1 region would be in most cases sufficient to characterize molecular variants. Overall targeted sequencing on recombinant junctions is a valid method of characterising isolates into molecular groups and an alternative method to whole genome sequencing or other molecular methods, where strain diagnostics are important.

2.4.4 The genomic diversity of PVY^N variants in Scotland

Sequencing of the R1, R2 and R3 regions of the genome has revealed a large degree of homogeneity between PVY isolates. They cluster in their large majority within the EU-NTN clade, which represent ca.76% of PVY^N isolates analysed. The NA-NTN and the N groups each make up 10-13% of the PVY^N population. As yet no tuber necrosis has been from Scottish seed potatoes; however, it would appear that the most prevalent strains present in the country fall in to the molecular group thought to be responsible for this disease. This is in line with findings across Europe, the United States of America, Asia and South Africa (Visser & Bellstedt, 2009, Rigotti *et al.*, 2001) where recombinant PVY strains are now dominant and have completely displaced non-recombinant PVY^O and PVY^N. All three recombination junctions could be

used for classification of isolates, the phylogenetic trees of the different proteins that were included in these regions show that there is variation in mutation rates between different proteins. Some regions such as the CI and NIa display relative homogeneity between isolates, whereas the VPg appears to be more variable. A possible explanation for this is that the VPg is the target of resistance genes in solanaceous species and therefore might be subject to strong selective pressure to overcome this type of resistance. Ayme *et al.* (2006) reported that virulent PVY isolates that overcome *pvr*^{2} resistance in pepper, as a result of substitutions within the VPg gene.

2.4.5 Genetic determinants of PVY necrosis in tobacco and potato: Analysis of PVY isolate 10088 a rare example of PVY^{E} strain

Analysis of the clustering isolates on the basis of genome sequence alone will not accurately reflect their biological properties as isolates with high levels of sequence identity have been shown to have major changes in their biological properties Barker *et al*. (2009). The definition of PVY strain require assessment of their pathogenicity in particular their ability to elicit hypersensitive response (HR) on potato cultivars harbouring either Nc, Ny or Nz resistance genes and vein necrosis on tobacco (Singh *et al.*, 2008). This study is described in Chapter 3. From all the PVY^{N} isolates selected, isolate 10088 did not trigger vein necrosis or HR on potato cultivars. Consequently, isolate 10088 was accordingly identified as PVY^{E} (see chapter 3 for further details). Sequencing data revealed that isolate 10088 clustered within the

molecular $PVY^{EU\text{-}NTN}$ clade. From the sequencing of the R1 and R2 and R3 regions of the PVY^{E} genome it would appear that PVY^{E} has a similar genetic structure to the EU-NTN's in all three regions. This implies that PVY^{E} isolates are rare but present in Scottish field populations. Isolate 10088 is molecularly related to $PVY^{EU\text{-}NTN}$ and does not trigger vein necrosis in tobacco such as PVY-MON which belong as well to the $PVY^{EU\text{-}NTN}$ group and is defined as a PVY^{E} strain (Galvino-Costa *et al*., 2012). Contrastingly, SASA61 (Barker *et al.*, 2009) belongs to the $PVY^{NA\text{-}NTN}$ group, suggesting that failing to elicit vein necrosis is not specific to a unique molecular group. The highest level of amino acid differences were between SCRI-O and SASA61. However both isolates illicit mosaic on tobacco, implying that the molecular determinants for vein necrosis are more complex than published literature may suggest (Tribodet *et al.*, 2005; Hu *et al.*, 2009)

Many authors have tried to locate the genetic determinants of vein necrosis in tobacco. Jacquot *et al* 2005 identified a single nucleotide polymorphism which they hypothesised could be used to molecularly determine whether an isolate would produce vein necrosis in tobacco or not, and by this infer the PVY strain type to be either PVY^{N} or PVY^{O}. They identified a single nucleotide change from A to G at nucleotide 2213 located within the HC-Pro (Glais *et al*., 2002). Hu *et al*. (2009b) also identified a single nucleotide polymorphism in the C-terminal region of the HC-Pro resulting in an amino acid change in the PVY^{NTN} type isolate L26. They suggest that this single change is responsible for the production of vein necrosis in tobacco plants. Faurez *et al* 2012

observed that two tobacco vein necrosis determinants have previously been identified in the HC-Pro K400 and E419, however they acknowledge that non necrotic isolates have also been reported as having these. The identification of PVY^{N} pathogenetic determinants was addressed by swapping genomic regions of the necrotic $PVY^{N\text{-}605}$ with a non-necrotic $PVY^{O\text{-}139}$ isolates. A series of $PVY^{N/O}$ chimeras and site selected mutations were constructed to test the involvement of different parts of the PVY genome. The analysis of both the genomic characteristics and biological properties of these mutants suggested the involvement of the two previously described determinants in the induction of vein necrosis in PVY isolates. Along with this they also identified N339 within the HC-Pro and two additional regions in the CI protein and the NIa region as having an effect.

In this study, of the PVY isolates that fail to elicit vein necrosis isolate 10088 (PVY^{E}) and SASA61 do not display nucleotide difference with other PVY^{N} (eliciting vein necrosis) at the proposed vein necrosis SNPs along the genome. No consensus in amino acid changes in either the HC-Pro or within the CP or NIa, associated with mosaic or vein necrosis symptoms in tobacco could be found. This implies that the published SNPs are not in all cases the molecular determinant of vein necrosis. One can argue that within a molecular group, where closely related isolates differ in their ability to trigger symptoms in tobacco (i.e. isolates 10088 and 9681 from the EU-NTN group) these may involve specific determinants without being necessarily shared with other isolates that belong to a different molecular groups (i.e. SASA61 and RRA1

from the NA-NTN group). Further studies involving mutational studies are required to define these determinants.

2.4.6 PTNRD and relation to molecular structure

Perhaps even more challenging to the molecular biologist than identify the molecular determinants of vein necrosis in tobacco is the identification of genetic determinants associated with PTNRD in potato. PVY^{N} and PVY^{NTN} are notoriously difficult to characterize without the use of time consuming bioassays (Kogovsek *et al*., 2008). The nucleotide sequence responsible for PTNRD development has not yet been identified. Along with the genomic characteristics of the virus PTNRD development is dependent on susceptibility of potato cultivar, the pathogenicity and environmental factors (Le Romancer and Nedellic, 1997). The initiation of the necrotic symptoms associated with PTNRD is believed to involve several stress related genes including heat shock and wound response (Pompe-Novak *et al*, 2006). In order to identify the molecular determinants of PTNRD it is first necessary to identify isolates that do not produce PTNRD in susceptible cultivars in optimal conditions and compare the molecular structure with isolates that easily produce PTNRD. Chapter 3 will assess how different cultivars react to different PVY isolates and whether it might be possible to use some of these isolates to determine the molecular determinants for PTNRD induction.

2.5 Conclusions

An assessment was made of field isolates submitted during the 2009 field inspections that were identified as PVY^N using ELISA. To ensure that the monoclonal antibodies were still suitable for detecting all Scottish PVY isolates a polyclonal survey of all samples submitted from field inspections was also undertaken. This survey revealed that all Scottish PVY isolates could currently be accurately detected using SASA monoclonal antibodies. Of the PVY^N isolates in Scotland the majority were molecularly characterised as EU-NTN. However, about a tenth were either NA-NTN or N variants. This comprehensively shows that the EU-NTN is not only present but dominates PVY^N in Scotland and it is perhaps the success of this group which has led to a rise in the PVY^N occurrence over the past decade. We have demonstrated that it is possible to use targeted sequencing over recombinant junction to accurately characterise isolates to strain groups which could reduce the need for full length sequencing. Our work also demonstrated that the hunt for the molecular determinants of both tobacco and tuber necrosis is complex and that SNP analysis may not always be a suitable method to determine the biological characteristics of an isolate.

Chapter 3 - Pathogenicity of PVY^N variants: leaf symptoms and Potato Tuber Necrotic Ringspot Disease (PTNRD)

3.1. Introduction

PVY exists as a complex of strains which can be distinguished on the basis of their biology (symptoms they elicit on indicator plants), serology and genome analysis. PVY strains were traditionally divided into three major groups: the ordinary (PVY^O), the stipple streak strain (PVY^C leaf drop of potato,) and PVY^N (veinal necrosis on tobacco) (De Boxk and Huttinga, 1981) according to their ability to elicit hypersensitive response in potato cultivars. These cultivars harbour corresponding dominant N resistance genes (Tain & Valkonen, 2013; Singh *et al*., 2008), which can be used to define five distinct PVY strain groups: PVY^O, PVY^N, PVY^C, PVY^Z and PVY^E (Singh *et al*., 2008, Kerlan *et al*., 2011).

Table 3.1: Genetic background of potato cultivars used for the biological characterisation of PVY and the type of the response elicited allowing the definition of PVY strain (Singh *et al.*, 2008).

	PVY^C	PVY^O	PVY^N	PVY^Z	PVY^E
King Edward (*Nc:ny:nz*)	HR	S	S	S	S
Duke of York (*Nc:ny:nz*)	HR	S	S	S	S
Pentland Crown (*nc:Ny:nz*)	S	HR	S	S	S
Maris Bard (*Nc:Ny:Nz*)	HR	HR	S	HR	S
Pentland Ivory (*Nc:Ny:Nz*)	HR	HR	S	HR	S
Tobacco	S	S	VN	S	S

HR: Hypersensitive Response (a form of localized cell death associated with resistance), S: susceptible (absence of local and systemic HR, systemic movement and elicitation of mosaic symptoms). VN (vein necrosis).

The ability to distinguish between different strains of the virus is a pre-requisite to the understanding of PVY^N population dynamics, evolution and pathogenicity. Currently there is an inherent difficulty in adopting a unified nomenclature for classifying PVY strains based on biological and molecular features making PVY strain classification difficult to harmonize (Kerlan *et al.*, 2011). Due to the high level of variation within PVY isolates is important to note that no single PVY detection method can guarantee correct diagnosis and typing of a PVY isolate (Galvino-Costa *et al.*, 2012).

Seed potato crops in Scotland are monitored for leaf symptom development throughout the growing season and are officially inspected at least twice for virus diseases. Inspectors are trained to visually identify the 30 most commonly grown potato cultivars in Scotland and to recognise mild and severe mosaic in these different cultivars. There are strict tolerances set for mild and severe mosaic symptoms for each seed potato grade based on the generation of the crop. The percentage of plants affected by either mild or severe mosaic symptoms within the crop is assessed visually. As inspection relies on the visual detection of virus symptoms, the ability of a virus isolate to elicit symptoms on a given variety can affect both the roguing (removal of symptomatic plants in the field) and the downgrading of crops (Ragsdale *et al*,

2001). PVY^N is known to elicit less severe symptoms on potato than PVY^O (Weidmann, 1988) for this reason it is possible where symptoms are particularly mild that plants infected with PVY^N might go undetected during inspections. It is therefore useful to evaluate leaf symptom severity elicited by different PVY^N (molecular and biological) variants.

Tuber symptoms induced by PVY represent an important threat to the potato industry. Potato tuber necrotic ringspot disease (PTNRD) is characterised by the appearance of necrotic rings on the surface of the tuber and deepening (sinking) of the necrotic rings at later stages of development depending on symptom severity (Boonham *et al.*, 2002). PTNRD was first reported in Hungary in the 1980s (Beczner *et al.*, 1984) and reached epidemic levels in many European countries during the 1980s (Le Romancer and Nedellic, 1997). The PVY strain thought to be responsible for tuber necrosis was a variant of PVY^N and so named PVY^N tuber necrosis or NTN (Le Romancer *et al*., 1994) and this variant was believed to be a recombination between PVY^O and PVY^N (Glais *et al*., 2002). Other than viral strain, the cultivar sensitivity and environmental factors are likely to be influential in PTNRD development. PTNRD symptoms are largely influenced by the length of tuber storage and the storage temperature (Le Romancer and Nedellec, 1997). Contrastingly, long-term cold storage has been shown to suppress PTNRD (Dolnicar *et al*., 2011). Therefore, PTNRD development is likely to be the result of the complex interactions between the genetic background of a potato cultivar, the

nature of a PVY^{N} strain and environmental factors (Le Romancer and Nedellec, 1997).

With new recombinant isolates of $PVY^{EU\text{-}NTN}$ replacing non recombinant PVY^{N} and PVY^{O} types in Scotland and throughout Europe, there is a threat that these may lead to reduction in both yield and tuber quality while producing mild foliar symptoms.

This chapter describes the characterization of a subset of PVY field isolates using a combination of serological, biological and molecular methods. All PVY isolates submitted to the SASA Virology laboratory originated from symptomatic seed potato plants identified at growing crop inspection. PVY isolates were subsequently characterized serologically by DAS-ELISA using monoclonal antibodies to discriminate them either as $PVY^{O/C}$ or as PVY^{N}. Further, passage inoculation of DAS-ELISA PVY^{N} positive isolates in tobacco was undertaken to assess whether these isolates elicit either vein necrosis (characteristic of PVY^{N} biotype) or mosaic (characteristic of PVY^{E} or PVY^{Z} biotypes), followed by molecular characterisation by targeted partial sequencing of known recombinant regions (R1, R2 and R3) within the PVY genome as described in Chapter 2. A subset of PVY isolates representing each of the molecular clades $PVY^{EU\text{-}N}$, $PVY^{NA\text{-}NTN}$ and $PVY^{EU\text{-}NTN}$ were selected and their pathogenicity further characterised by assessing the physiological responses of potato cultivars harbouring a known set of PVY resistance genes *Nc*, *Ny* or *Nz* (Singh *et al.,* 2008). The pathogenicity of this representative

subset of PVY^N isolates in their ability to trigger leaf symptoms and PTNRD on a range of commonly grown potato cultivars will be presented, as a means for evaluating the potential threat they might pose to the potato industry and certification scheme.

3.2. Methods

3.2.1. Sampling of PVY^N field isolates

All samples submitted by the RPID (Rural Payment Inspection Directorate, Scottish Government) field inspectors from the 2009 season that were DAS-ELISA positive for PVY^N were inoculated into tobacco plants *Nicotiana tabacum* cv. White Burley and *Nicotiana benthamiana*. Leaves of these plants were freeze dried using an Edwards Modulyo 4K freeze-dryer (Crawley, UK) labelled and stored for use prior to the start of this project. The isolates that were used for the following work were only those identified as PVY^N by ELISA.

3.2.2 Biological characterisation of PVY isolates using tobacco

Biological characterisation of PVY^N isolates involved firstly inoculating tobacco plants *N. tabacum* cv. White Burley and *N. benthamiana*. Five week-old plants were mechanically inoculated using infectious sap by rubbing leaves using carborundum as an abrasive (grit size 400). Sap for inoculation

was prepared by grinding the freeze-dried material in a 50 mM Na_2HPO_4/ KH_2PO_4 pH7.5 buffer (2 w/v or at least 2 mL) using a Bioreba AG Homex 6 homogeniser (Bioreba, Switzerland). PVY isolates were propagated and maintained in *N. benthamiana* and *N. tabacum* cv. White Burley. Inoculated plants were grown in a temperature controlled glasshouse (20^{o}C for 16 h and 15^{o}C for 8 h overnight). Plants were inspected twice weekly for symptom development and symptoms were recorded for each isolate. Vein necrosis or mosaic appeared within 10-15 days post-inoculation. Isolates that failed to trigger symptoms on tobacco initially were reinoculated into a new set of tobacco plants. However, approximately 95% of these isolates failed to produce any symptoms during re-infection so an additional 60 PVY^N field isolates intercepted during the 2010 growing crop season were also included.

All isolates that failed to produce vein necrosis despite identification as PVY^N type by DAS-ELISA were recorded and re-inoculated in to *N. tabacum* cv. White Burley and *N. benthamiana* to confirm the symptoms produced. For all isolates that induced symptoms in either *N. tabacum* cv. White Burley or *N. benthamiana*, symptomatic leaves were collected. At least one symptomatic leaf from each isolate was homogenized using a Bioreba AG Homex 6 homogeniser (Bioreba, Switzerland) in 2 mL 50 mM Na_2HPO_4/ KH_2PO_4 pH 7.5 buffer. The sap produced was stored at -20°C for future inoculations.

3.2.3 Mechanical inoculation of potatoes for the biological characterisation of PVY isolates

Infectious sap for each of the PVY isolates was prepared using leaves of infected *N. benthamiana* and *N. tabacum* cv White Burley plants. At 2 weeks post inoculation, symptomatic leaves were harvested and homogenised in an equal volume (w/v) of 50 mM Sorensens phosphate buffer pH7.2. The selected PVY isolates were serologically positive for PVY^{N} and included three molecularly defined $PVY^{EU\text{-}NTN}$ isolates, two of which induced vein necrosis on tobacco (10057 and 9592) and one that produced mosaic symptoms (10088). Two isolates from the molecular $PVY^{NA\text{-}NTN}$ clade (9737 and 9561) and one isolate belonging to the $PVY^{EU\text{-}N}$ clade (9552) were also selected following partial sequencing as described in Chapter 2.

Potato cultivars were grown from virus-free micropropagated *in vitro* material, which were planted in Jiffy pots for 2 weeks and then transferred to 6 inch pots and grown for a further 4 weeks. The two lowest leaves were manually rub-inoculated using coarse grit carborundum (size 400) and 50 µL of infectious sap per leaf at 4 weeks post-planting. Four biological replicates per cultivar were challenged with each PVY isolate. Non-inoculated control plants were maintained alongside infected cultivars. The potato varieties selected for biological indexing were King Edward, Duke of York, Pentland Crown, Maris Bard, and Pentland Ivory (Singh *et al.*, 2008). Symptoms were recorded twice weekly after an initial period of 18 days. Leaf symptoms were

scored on a scale of 0 to 5 (0 - asymptomatic, 5 - most severe mosaic and crinkled symptoms).

3.2.4. Sampling of potato leaves

At 21 and 38 days post inoculation the third leaf from the apex of each plant was removed and placed in a Bioreba bag (Bioreba, Switzerland). Samples were tested using monoclonal antibodies by DAS-ELISA as described in chapter 2 in order to detect PVY presence and assess PVY concentration.

3.2.5. Inoculation of potato plants to assess PTNRD development in a range of potato cultivars

In order to investigate potential PTNRD development in 16 of the most commonly grown seed potato varieties in Scotland, inoculum was prepared as described in section 2.2.4. Potato cultivars were also categorised on the basis of their known resistance characteristics towards PVY^{O}, either with high (Disease Index = 7-9), medium (Disease Index = 4-6) or low (Disease Index = 2-3) resistance levels (source http://varieties.potato.org.uk/menu.php). The potato varieties with a low resistance to PVY^{O} were Maris Piper, Maris Peer, Estima, Valor and King Edward. The potato varieties with a medium resistance to PVY^{O} were Lady Rosetta, Pentland Dell, Marfona, Saxon and Maris Bard. The potato varieties with a high level of resistance to PVY^{O} were Hermes, Nadine, Markies, Cara, Melody and Picasso. The PVY isolates used were PVY^{O} (DV71), $PVY^{NA\text{-}NTN}$ (DV69), $PVY^{EU\text{-}NTN}$ (DV76) which are from

a historic diseased variety collection maintained at SASA and $PVY^{EU\text{-}N}$ (9552) identified in Chapter 2. Cultivars were grown in optimal temperature conditions for PTNRD development (Le Romancer and Nedellec, 1997) at a constant 20^{o}C in a glasshouse (16h photoperiod). Four plants of each variety per isolate combination were grown for both the glasshouse and in the field trials. Plants were inoculated at 4 weeks growth using 50 µL of inoculum on two lower leaves of each plant. Field-grown plants were treated with blight spray and insecticide and grown in separate plots to reduce the potential of cross-infection by others PVY isolates.

3.2.6. Assessment of PTNRD symptoms

PTNRD was assessed on the previously mentioned cultivars used for biological characterisation including also Maris Piper (most commonly grown cultivar in Scotland) and Nadine (most PTNRD susceptible cultivar grown in Scotland) over a 3 year period. Along with the six PVY^{N} isolates used for biological typing, Maris Piper and Nadine were also inoculated with a $PVY^{N\text{-}Willga}$ and a PVY^{O} isolate. In the first year the plants used for these trials originated from *in vitro* micropropagated explants. Ahloowalia (1994) demonstrated that micropropagated plants produce miniature tubers after 70 to 115 days of growth. Many of these tubers produced were between 9 and 15mm in diameter, so in order to produce larger tubers, potato plants were grown for 140 days. In the second and third years of the trial, tubers from infected plants from the previous year were re-planted. Four tubers of each cultivar per isolate combination were planted in the glasshouse, while in year 2 six were planted in a field plot. In the field trial, cultivars infected with the same isolates were planted alongside and two drills were left between each of the plots of plants infected by a different isolate to minimize cross-infection. In addition, the plots were covered with insect proof netting in order to prevent the transmission of virus by aphids. The field trial suffered strong blight infection in year 2, and as a consequence, it was decided not to pursue the field trial for a third season and to maintain only the glasshouse trial for the third season.

For all trials, haulms were cut 2 weeks before harvest to allow the skin to set. Tubers were assessed for symptoms directly after harvest ("No storage") and at 3 months after storage at permissive conditions (in the dark at the constant temperature of 20°C) (Le Romancer and Nedellec, 1997).

In order to record and assess the severity of PTNRD symptoms each tuber was given a score (PTNRD index) ranging from 0 to 4 (Figure 3.1) from the absence of symptoms to the severe ringspot necrosis symptoms covering more than 70% of the tuber surface.

- PTNRD index = 0: symptomless
- PTNRD index = 1: one small lesion not forming a ring (blister)
- PTNRD index = 2: one clear sunken necrotic ringspot
- PTNRD index = 3: ringspot or blisters covering 40% - 70% of tuber surface
- PTNRD index = 4: ringspot lesions covering more than 70% of tuber surface

The PTNRD index of a cultivar infected by a given PVY isolate was calculated by averaging individual score from each tuber per plant for all plants tested.

Figure 3.1 PTNRD symptoms index scale. Example of PTNRD index from 0 to 4 is illustrated by displaying a range of symptomatic tubers from the cultivar Nadine as previously described.

3.3 - Results

3.3.1. Selection of PVY^N field isolates

A total of 259 leaf samples submitted in 2009 tested positive for PVY^N by DAS-ELISA. To maximise the diversity of PVY^N field isolates, DAS-ELISA positive PVY^N leaf samples were selected from a wide range of geographical regions covering all seed potato growing areas in Scotland (Central, Grampian, Highlands and South-East) from 66 different potato varieties. The highest numbers of cases were recorded from Maris Piper, which was also the most cultivated seed crop in Scotland in that year. This demonstrates that PVY, particularly PVY^N can infect a very broad range of potato varieties, unlike other potyviruses such as PVA which only infects a few susceptible cultivars (data not shown).

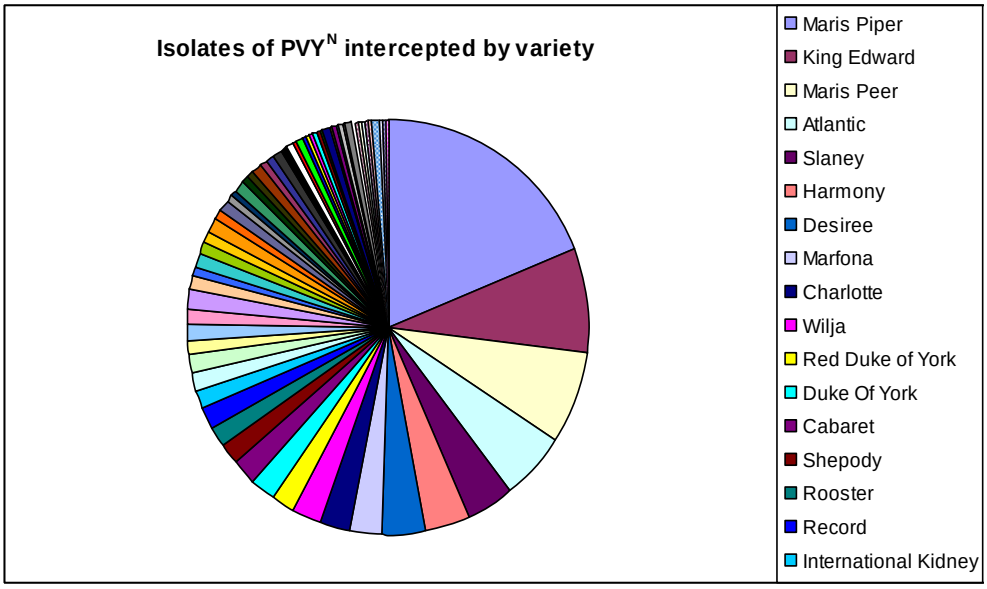


Figure 3.2 Percentage of PVY^N cases by cultivar (field survey 2009). The identity of the 17 first cultivars with the highest number of PVY^N cases intercepted is reported (SASA SPCS Virus testing data 2009).

3.3.2 Biological indexing of PVY^N field isolates using tobacco

The ability of PVY^N isolates to cause the development of either vein necrosis (VN) or mosaic in *N. tabacum* cv. White Burley was assessed. As all of the isolates had been stored from their original freeze-dried potato leaf sample for a period of 3 to 6 months, there was the possibility of loss of infectivity of the PVY^N isolates prior to inoculation into tobacco plants. Overall 259 isolates were inoculated to tobacco, and of these 111 (43%) had still retained their infectivity and elicited typical viral symptoms. Most of them (95%) induced vein necrosis in tobacco cv. White Burley. For any isolates that produced

symptoms on *N. benthamiana* but failed to produce vein necrosis in tobacco cv. White Burley, a second inoculation was performed to confirm the phenotype. In total there were five isolates that fell within this category. The symptomatic leaves produced by these isolates were photographically recorded and retested using DAS-ELISA. Of these five isolates, one was identified serologically as PVY^{O}, one tested negative for PVY and the other three were all positive for PVY^{N} despite inducing mosaic as opposed to vein necrotic symptoms. The isolate that tested negative for PVY produced upwards curling of the leaves on *N. benthamiana*, rather than the stunting and crinkling normally encountered in PVY infected plants. This isolate was tested by DAS-ELISA using MAbs raised against 11 main potato-infecting viruses and found to be ELISA positive for PVX and was therefore removed from the study.

Figure 3.3 Symptoms elicited by two PVY isolates in *Nicotiana tabacum* cv. White Burley. Mosaic symptoms upon infection by PVY isolate 10088 (left panel). Typical vein necrosis symptoms upon infection by PVY isolate 9929 (right panel) at 14 days post-inoculation (dpi).

3.3.3 Biological characterisation of PVY isolates on potato

Resistance specificity of differential potato cultivars harbouring PVY-specific resistance genes (*Nc*, *Ny*, *Nz*) allows the classification of PVY isolates into biological strain groups (Singh *et al*., 2008, Table 3.1). The potato cultivars used to test for the above-mentioned resistance characteristics are King Edward, Duke of York, Pentland Crown, Maris Bard, and Pentland Ivory. Six representative PVY^{N} isolates were selected for further characterisation on the basis of their genomic sequence and the symptoms elicited on tobacco plants: (*i*) three isolates that cluster within the $PVY^{EU\text{-}NTN}$ clade, two of which elicited vein necrosis (9529, 10057) and one which induced mosaic (10088) on tobacco; (*ii*) two isolates that cluster within the $PVY^{NA\text{-}NTN}$ clade (9561, 9737); and (*iii*) one isolate that clusters within the $PVY^{EU\text{-}N}$ clade (9552). Following infection of the potato cultivars listed in Table 3.1, (Duke of York substituted for Desiree), none of the PVY isolates triggered a hypersensitive response in the cultivars tested (Table 3.2) indicating that these cultivars are therefore susceptible to all isolates tested. This means that the five PVY isolates tested that elicited vein necrosis in tobacco belong to the strain group PVY^{N} while the PVY isolate (10088) that elicited mosaic as opposed to vein necrosis in tobacco belongs to the strain group PVY^{E}

Table 3.2: Symptoms induced by selected PVY field isolates (9552, 10057, 10088, 9737, 9561 and 9529) in five *Solanum tuberosum* cultivars and tobacco cultivar White Burley.

Isolate	Molecular Type (R1 RJ)	King Edward (*Nc*)	Pentland Ivory (*Nc:Ny:Nz*)	Maris Bard (*Nc:Ny:Nz*)	Duke of York (*Nc*)	Pentland Crown (*Ny*)	Tobacco cv White Burley	Biological Type
9552	**EU-N**	mo +++	mo ++	mo ++	mo ++	mo +	VN	**PVY-N**
10057	**EU-NTN**	mo ++	mo ++	mo +++	mo ++	mo +	VN	**PVY-N**
10088	**EU-NTN**	mo ++	mo +	mo ++	mo ++	mo ++	mo +++	**PVY-E**
9737	**NA-NTN**	mo ++	mo ++	mo ++	mo ++	NS	VN	**PVY-N**
9561	**NA-NTN**	mo, crk +++	mo +++	mo ++	mo+++	NS	VN	**PVY-N**
9529	**EU-NTN**	mo, crk +++	mo, crk +++	mo, crk +++	mo ++	mo +	VN	**PVY-N**

Symptoms: mo (mosaic), crk (crinkling), nec (necrosis), NS (non-symptomatic), VN (vein necrosis)
Intensity of symptoms: faint (+), intermediate (++) and severe (+++)

3.3.4 Leaf symptom development on potato cultivars infected by selected PVY isolates

Symptom development on potato cultivars infected for biological characterisation was regularly assessed between 18 dpi (days post inoculation) to 38 dpi.

Table 3.3 Symptom expression in potato cultivars between 18 to 38 dpi. Disease index (DI) score range from 0 (symptomless) to 5 (severe mosaic and crinkle). Four plants per cultivar were tested for each of the six PVY isolates. Results are the range of scores from the 24 plants assessed per cultivar.

	King Edward	Maris Bard	Duke of York	Pentland Ivory	Pentland Crown
18 dpi	3-4	2-3	3-4	1-2	0-1
21 dpi	2-3	2-3	3-4	2-3	1-2
24 dpi	3-4	3-4	3-4	2-3	1-2
26 dpi	4-5	3-4	3-4	2-3	0-1
31 dpi	4-5	4-5	3-4	2-3	1-2
34 dpi	3-4	3-4	3-4	1-2	1-2
38 dpi	3-4	4-5	3-4	2-3	1-2
Overall	**3-4**	**3-4**	**3-4**	**2-3**	**1-2**

For all the cultivars tested, systemic mosaic symptoms were visible by 18 dpi. Symptom severity differed between cultivars. For King Edward by 18dpi leaf symptoms were well pronounced. There was some variation in the severity of mosaic symptoms triggered which was most acute by 31 days. For Maris Bard symptom expression generally increased until 38 dpi. The Duke of York cultivar was consistently expressing leaf symptoms with a DI of 3-4 across the assessment period. Symptoms on the Pentland Ivory cultivar remained

relatively stable during the assessment period with a disease index of 2 while the Pentland Crown cultivar displayed an average DI of 1. Overall there were different levels of symptom intensity between cultivars ranging to the most severe for King Edward and Maris Bard (DI maximum of 4-5) to milder symptoms in Pentland Crown (DI maximum of 1-2). Figure 2.4 displays a range of the most representative symptoms recorded across a range of selected cultivars.

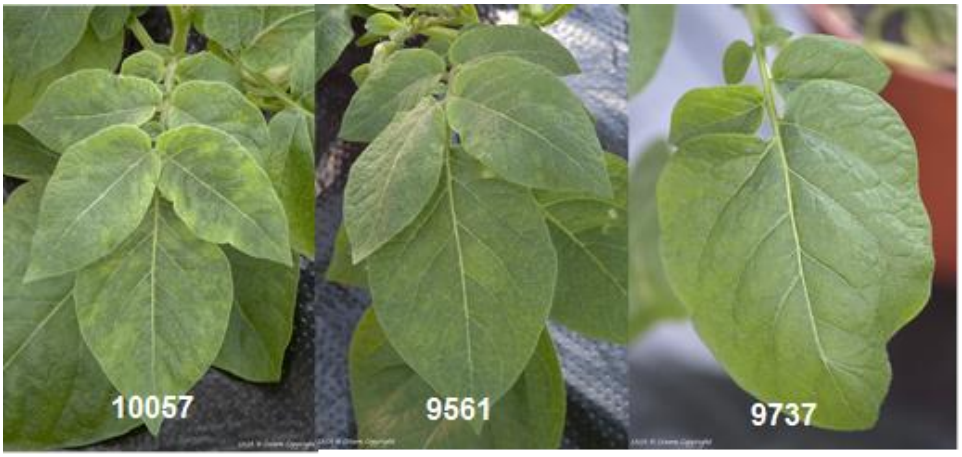


Figure 3.4a Typical leaf symptoms observed on infected cultivar Pentland Ivory. Left panel: severe mosaic with a disease index (DI) of 4, middle panel mild mosaic DI 2 and right panel symptomless with a DI of 0. The corresponding isolate numbers are recorded at the base of each panel.

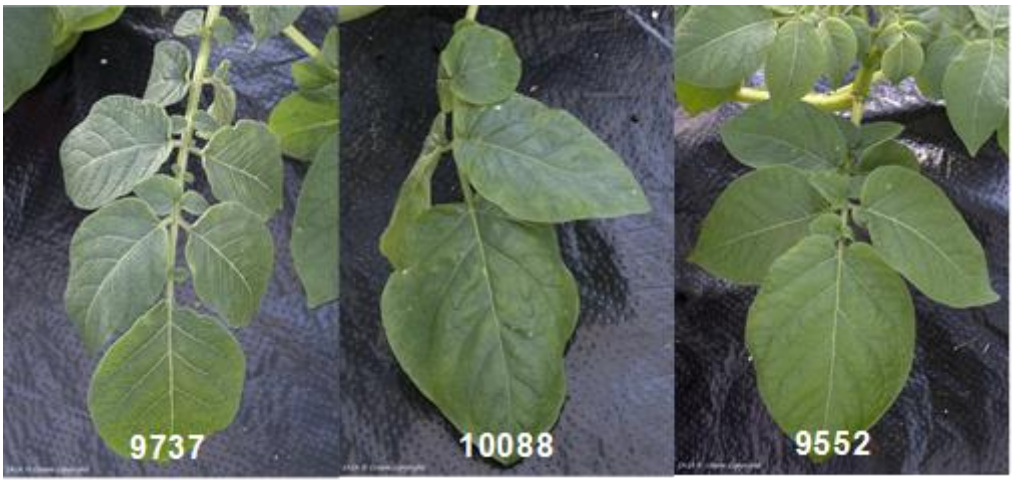


Figure 3.4b Typical leaf symptoms observed on infected cultivar Pentland Crown. Left panel: Mild mosaic with a disease index (DI) of 1, middle panel mild mosaic DI 2 and right panel symptomless with a DI of 0. The corresponding isolate numbers are recorded at the base of each panel.

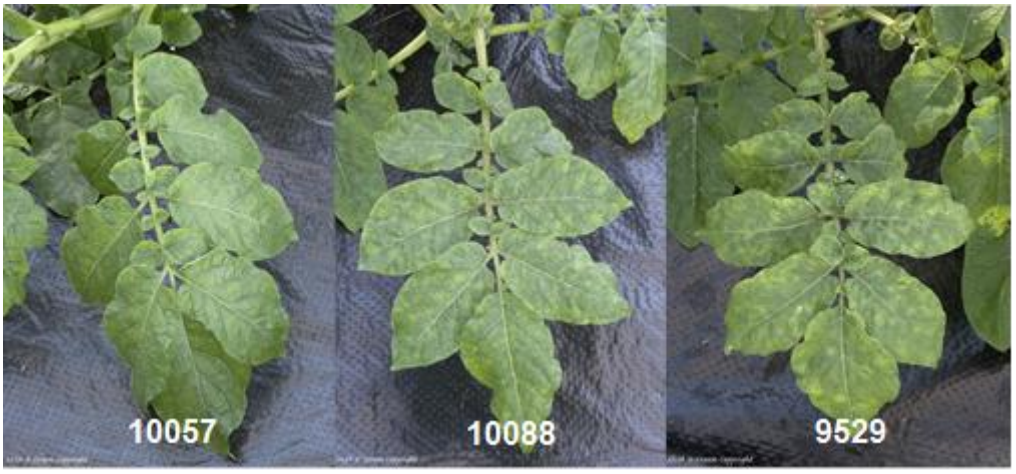


Figure 3.4c Typical leaf symptoms observed on infected cultivar Duke of York. Left panel: Mild mosaic with a disease index (DI) of 1, middle panel severe mosaic DI of 3 and right panel severe mosaic with a DI of 4. The corresponding isolate numbers are recorded at the base of each panel.

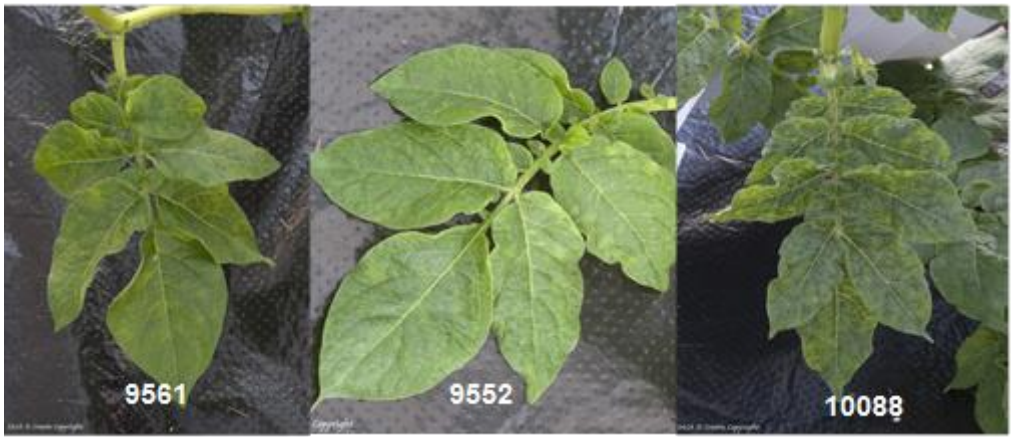


Figure 3.4d Typical leaf symptoms observed on infected cultivar King Edward. Left panel: Mild mosaic with a disease index (DI) of 2, middle panel mild mosaic DI of 2 and right panel severe mosaic with a DI of 5. The corresponding isolate numbers are recorded at the base of each panel.

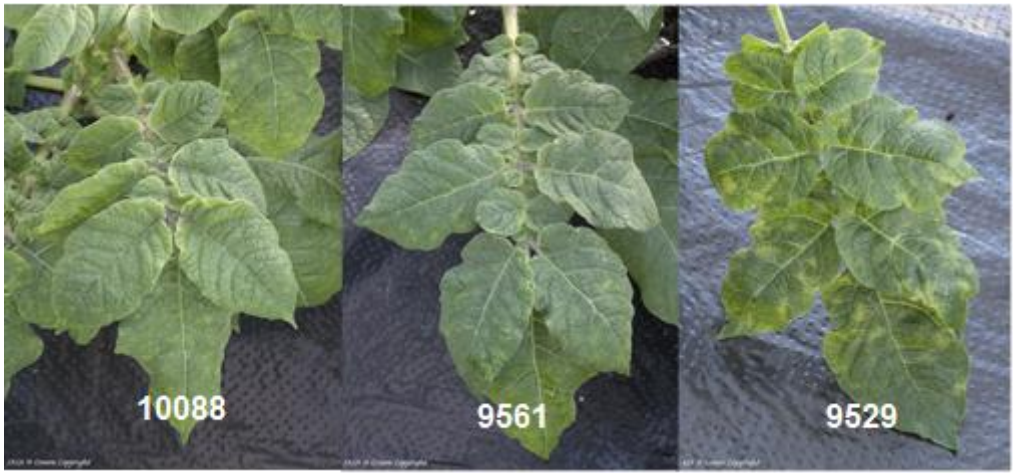


Figure 3.4e Typical leaf symptoms observed on infected cultivar Maris Bard. Left panel: Mild mosaic with a DI=1, middle panel mild mosaic DI=2 and right panel severe mosaic with a DI= 3. The corresponding isolate numbers are recorded at the base of each panel.

3.3.5 Assessment of leaf symptom severity in relation to virus titre

To evaluate more accurately the aggressiveness of PVY isolates, PVY concentration was determined in leaves in relation to disease symptom severity (at 21 dpi) for each isolate-cultivar combinations. Results are presented in Table 3.4. This dataset allowed assessment of symptom severity in relation to virus titre within the same leaf material, with the view to evaluate the aggressiveness of a PVY isolate against these five potato cultivars.

Table 3.4 Symptom severity in relation to virus titre in leaves of five potato cultivars. Symptoms (Disease Index – DI) were scored and OD measured at 21 days post inoculation.

			King Edward (*Nc*)		Pentland Ivory (*Nc:Ny:Nz*)		Maris Bard (*Nc:Ny:Nz*)		Duke of York (*Nc*)		Pentland Crown (*Ny*)		Average per isolate	
Isolate	**Molecular group**	**Biological group**	OD	DI	OD	DI	OD	DI	OD	DI	OD	DI	**OD**	**DI**
9552	EU-N	PVY-N	0.68-0.86	3 - 5	0.20 - 0.33	2 - 3	0.74 - 0.98	2 - 3	0.74 - 1.02	3 - 4	0.41 - 0.61	0 - 2	**0.648**	**2.7**
10057	EU-NTN	PVY-N	0.77-0.99	3 - 4	0.15 - 0.58	2 - 4	0.78 - 0.96	3 - 4	0.48 - 0.79	3 - 4	0.55 - 0.66	0 - 2	**0.644**	**2.9**
10088	EU-NTN	PVY-E	0.15-0.35	1 - 4	0	1 - 2	0.16	1 - 3	0.14 - 0.14	1 - 4	0.39	2 - 3	**0.214**	**2.7**
9737	NA-NTN	PVY-N	0.7 - 1.1	1 - 3	0.35 - 0.51	1 - 3	0.52 - 0.84	2	0.72 - 0.81	3 - 4	0.21 - 0.7	0	**0.678**	**1.7**
9561	NA-NTN	PVY-N	0.7 - 1.1	4	0.11 - 0.47	3 - 4	0.44 - 0.81	2 - 3	0.12 - 0.58	4	0.56 - 0.66	0 - 1	**0.557**	**2.9**
9529	EU-NTN	PVY-N	0.9 - 1.0	4	0.22 - 0.34	2 - 4	0.69 - 1.14	4 - 5	0.75 - 1.12	4	0.64 - 0.98	1 - 3	**0.820**	**3.5**
		Average per cultivar	**0.72**	**3.2**	**0.315**	**2.8**	**0.696**	**2.9**	**0.633**	**3.5**	**0.597**	**0.9**		

DI= Disease Index in leaves (individual plants were scored for symptoms on a scale on 1 -5 results are the range of 4 replicates per cultivar / isolate combination).

OD = Optical Density (virus titre was measured by DAS- ELISA at OD_{405} results are the range of 4 replicates per cultivar / isolate combination)

An isolate could be defined as “aggressive” when a high disease index (DI) was observed for a relatively similar or lower virus titre in comparison to other isolates. An isolate was classified as “mild” when a lower disease index was observed for a relative similar or higher virus titre in comparison to other isolates. Therefore, isolates 9561 and 10088 could be classified as aggressive/severe (similar to low OD for a high DI). Contrastingly, isolate 9737 could be classified as mild (similar OD for a low DI value). In addition, this dataset allows us to define characteristics of the selected potato cultivars ranging from highly susceptible (*i.e.* expressing obvious virus symptoms and high DI index >2.5 for a high or similar virus titre) to cultivars that support latent PVY infection (*i.e.* absence of, or very mild symptoms (DI<1) for a comparable virus titre). Thereby, cultivar Pentland Crown could be identified as a cultivar supporting latent infection of PVY (Table 3.4) as low DI was found and only symptomless to very mild symptoms could be seen on this cultivar. A Spearman’s rank correlation between virus titre and symptom severity by cultivar produced a rho value of 0.6 and a p-value of 0.35, and by isolate produced a rho value of 0.147 and p-value of 0.781. This would suggest there is no relationship between symptom severity and virus titre.

3.3.6 Assessment of Potato Tuber Necrotic Ringspot Disease (PTNRD) development

The cultivars Nadine, Maris Piper, Maris Bard, Pentland Crown, Pentland Ivory and Duke of York were infected with the previously described PVY

isolates. The cultivars chosen for this trial were those used for biological characterisation along with Maris Piper and Nadine. Table 3.5 reports the results of PTNRD severity observed in tubers over a three year trial period in glasshouse conditions.

Table 3.5a PTNRD symptom assessment of 3 generations of infected potato cultivars. 2010 (1st generation) 2011 (2nd generation with field results) and 2012 (3rd generation). Scoring is as described in 2.2.7 The PTNRD index presented was obtained by averaging indexes from all tubers per plant. Where N.A. results not available.

Cultivar	PVY Isolate	PTNRD index*							
	Biotype / Molecular group / Isolate code	Progeny tubers 1st generation		Progeny tubers 2nd generation		Progeny tubers 2nd gen. field		Progeny tubers 3rd generation	
		No storage	3 months storage	No storage	3 months storage	No storage	3 months storage	No Storage	3 Months
King Edward	N / EU-N / 9552	0.00	0.00	0.00	0.00	0	0	0.00	0.00
King Edward	N / EU-NTN / 9529	0.00	0.00	0.00	0.00	0	0	0.00	0.00
King Edward	N / EU-NTN / 10057	0.00	0.00	0.00	0.00	0	0	0.00	0.00
King Edward	E / EU-NTN / 10088	0.00	0.00	0.00	0.00	0	0	0.00	0.00
King Edward	N / NA-NTN / 9737	0.00	0.00	0.00	0.00	0	0	0.00	0.00
King Edward	N / NA-NTN / 9561	0.00	0.00	0.00	0.00	0	0	0.00	0.00
Maris Bard	N / EU-N / 9552	0.00	0.10	0.28	0.53	0	0	0.06	0.34
Maris Bard	N / EU-NTN / 9529	0.00	0.04	0.11	0.53	0	0	0.00	0.22
Maris Bard	N / EU-NTN / 10057	0.00	0.00	0.07	0.32	0	0	0.00	0.06
Maris Bard	E / EU-NTN / 10088	0.00	0.14	0.05	0.55	0	0	0.00	1.17
Maris Bard	N / NA-NTN / 9737	0.00	0.00	0.06	0.20	0	0	0.16	0.18
Maris Bard	N / NA-NTN / 9561	0.00	0.03	0.10	0.59	0	0	0.00	0.05
Duke of York	N / EU-N / 9552	0.00	0.03	0.00	0.08	0	0	0.00	0.00
Duke of York	N / EU-NTN / 9529	0.00	0.00	0.00	0.29	0	0	0.00	0.00
Duke of York	N / EU-NTN / 10057	0.00	0.05	0.00	0.04	0	0	0.08	0.20
Duke of York	E / EU-NTN / 10088	0.00	0.06	0.00	0.25	0	0	0.00	0.13
Duke of York	N / NA-NTN / 9737	0.00	0.00	0.00	0.25	0	0	0.08	0.08
Duke of York	N / NA-NTN / 9561	0.00	0.00	0.00	0.20	0	0	0.07	0.20
Pentland Ivory	N / EU-N / 9552	0.00	0.50	0.25	1.12	0	0.08	0.00	0.13
Pentland Ivory	N / EU-NTN / 9529	0.00	0.00	0.11	0.52	0	0	0.00	0.05
Pentland Ivory	N / EU-NTN / 10057	0.00	0.25	0.00	0.18	0	0	0.00	0.03
Pentland Ivory	E / EU-NTN / 10088	0.00	0.17	0.13	0.31	0	0	0.00	0.00
Pentland Ivory	N / NA-NTN / 9737	0.00	0.18	0.06	1.04	0	0.05	0.01	0.09
Pentland Ivory	N / NA-NTN / 9561	0.00	0.42	0.16	1.34	0	0.33	0.20	0.34
Pentland Crown	N / EU-N / 9552	0.00	0.72	0.25	0.75	0.03	0.47	0.00	0.61
Pentland Crown	N / EU-NTN / 9529	0.00	0.37	0.13	1.29	0	0.17	0.00	0.90
Pentland Crown	N / EU-NTN / 10057	0.00	0.07	0.00	0.62	0	0	0.00	0.00
Pentland Crown	E / EU-NTN / 10088	0.00	0.33	0.00	0.85	0	0.06	0.00	0.25
Pentland Crown	N / NA-NTN / 9737	0.00	0.16	0.08	1.25	0	0.33	0.00	0.00
Pentland Crown	N / NA-NTN / 9561	0.00	0.18	0.23	1.55	0	0.71	0.00	0.44
Maris Piper	N / EU-N / 9552	0.00	N.A.	0.00	0.00	0	0	0.00	0.00
Maris Piper	N / EU-NTN / 9529	0.00	N.A.	0.00	0.00	0	0	0.00	0.00
Maris Piper	N / EU-NTN / 10057	0.00	N.A.	0.00	0.00	0	0	0.00	0.00
Maris Piper	E / EU-NTN / 10088	0.00	N.A.	0.00	0.00	0	0	0.00	0.00
Maris Piper	N / NA-NTN / 9737	0.00	N.A.	0.00	0.00	0	0	0.00	0.00
Maris Piper	N / NA-NTN / 9561	0.00	N.A.	N.A	N.A.	0	0	N.A	0.00
Maris Piper	O / O / 9792	0.00	N.A.	0.00	0.00	0	0	0.00	0.00
Maris Piper	O / N-Wilga / 10766	0.00	N.A.	0.00	0.00	0	0	0.00	0.00
Nadine	N / EU-N / 9552	3.00	N.A.	No Growth	N.A.	No Growth	N.A.	N.A	N.A
Nadine	N / EU-NTN / 9529	3.65	N.A.	No Growth	N.A.	No Growth	N.A.	N.A.	N.A
Nadine	N / EU-NTN / 10057	2.98	N.A.	No Growth	N.A.	No Growth	N.A.	N.A	N.A
Nadine	E / EU-NTN / 10088	1.80	N.A.	No Growth	N.A.	No Growth	N.A.	N.A	N.A
Nadine	N / NA-NTN / 9737	3.59	N.A.	No Growth	N.A.	No Growth	N.A.	N.A	N.A
Nadine	N / NA-NTN / 9561	4.00	N.A.	4.00	4.00	No Growth	N.A.	0.00	3.00
Nadine	O / O / 9792	1.40	N.A.	No Growth	N.A.	No Growth	N.A.	N.A	N.A
Nadine	O / N-Wilga / 10766	0.18	N.A.	0.00	0.00	0	0	0.00	0.00

Table 3.5b mean values by cultivar and isolate from Table 3.5a

Cultivar / Isolate	No Storage	3 Month Storage
King Edward	0 (±0)	0 (±0)
Maris Bard	0.05 (±0.018)	1.04 (±0.071)
Duke of York	0.01 (±0.007)	0.11 (±0.024)
Pentland Ivory	0.03 (±0.019)	0.35 (±0.097)
Pentland Crown	0.04 (±0.019)	0.65 (±0.109)
Maris Piper	0 (±0)	0 (±0)
Nadine*	2.39 (±0.459)	n/a
9552 (EU-N)	0.06 (±0.028)	0.33 (±0.095)
9529 (EU-NTN)	0.04 (±0.013)	0.28 (±0.1)
10057 (EU-NTN)	0.01 (±0.007)	0.11 (±0.044)
10088 (EU-NTN/E)	0.01 (±0.009)	0.28 (±0.088)
9737 (NA-NTN)	0.03 (±0.012)	0.23 (±0.099)
9561 (NA-NTN)	0.05 (±0.021)	1.08 (±0.124)

Standard error values are included in brackets

*Only results from Nadine on first year's harvest are included.

In the second year of the trial, plants were also planted in the field and these results are included in Table 3.5. Symptoms were assessed according to Figure 3.1 where the maximum PTNRD Index of 4 for highly symptomatic tubers (full coverage of tuber with necrotic lesions) down to a minimum of 0 for tubers displaying no symptoms (symptomless). The PTNRD Index for each variety was calculated by averaging the score of all tubers in one plant, and subsequently averaged for all plants tested. All tubers were reassessed at 1

month post harvest, at this stage and a few tubers were beginning to develop mild PTNRD symptoms (blisters). At three months post harvest all tubers were scored (using the PTNRD Disease Index scale described in methods section 3.2.7). After storage at permissive conditions symptoms were observed in Maris Bard, Duke of York, Pentland Ivory and Pentland Crown.

In year one only Nadine was found to display PTNRD symptoms on harvest:

- These symptoms were observable following infection by all PVY isolates tested.
- Most of these tubers were severely affected reaching PTNRD DI of 2.98 and above.
- The lowest PVY^{N} PTNRD DI in Nadine was observed with isolate 10088 ($PVY^{E/EU-NTN}$) with only 1.8, the PVY^{O} isolate 9792 had a DI of 1.4 and the lowest DI was for the $PVY^{N-Wilga}$ isolate 10766 with a PTNRD DI of 0.18.
- PVY^{O} infected plants were small and symptomatic, suggesting that PVY^{O} which is not usually described as a strain associated with ringspot disease can also trigger PTNRD in a susceptible cultivar.
- Of the six serologically PVY^{N} isolates tested, five induced severe PTNRD symptoms with average scores ranging from 3-4 (maximum possible score of 4).

During the first year of the trial, Maris Piper and Nadine tubers were replanted immediately after treatment with gibberellic acid to promote rapid sprouting, explaining the lack of available data on PTNRD incidence after storage (Table 3.5).

In year one after storage, (of the five cultivars that were stored):

- Symptoms were seen in four cultivars (Maris Bard, Duke of York, Pentland Ivory and Pentland Crown) but these symptoms were far weaker than those induced in cv. Nadine.
- The most symptomatic was Pentland Crown with a maximum PTNRD DI of 0.72, then Pentland Ivory with a maximum PTNRD DI of 0.5, Maris Bard had a maximum PTNRD DI of 0.14 and Duke of York was the least symptomatic with a maximum PTNRD DI of 0.06.
- The only cultivars with tubers remaining symptomless after storage was King Edward.
- After storage, the isolate 9552 (PVY^{EU-N}) produced the most symptomatic tubers and isolate 10057 (PVY^{EU-NTN}) produced the least symptoms, but all isolates produced mild symptoms.

For the second year of the trial harvested progeny tubers from all infected potato plants were replanted:

- The majority of affected tubers from cultivar Nadine failed to produce a viable plant with the exception of Nadine progeny infected by PVY

isolate 9561, for which all tubers scored a maximum PTNRD DI of 4.0 both at harvest and after storage.

- For Maris Bard, Pentland Ivory and Pentland Crown, all displayed mild symptoms at harvest (maximum PTNRD DI ranging from 0.25 - 0.28).
- Cultivars King Edward, Duke of York and Maris Piper did not display PTNRD symptoms at harvest.
- After three months storage Pentland Crown tubers reached a maximum PTNRD DI of 1.55, Pentland Ivory 1.34 and Maris Bard 0.59 and Duke of York tubers were mildly symptomatic with a maximum of 0.29.
- The most severe PVY isolate was 9561 ($PVY^{NA\text{-}NTN}$) while the mildest was isolate 10057 ($PVY^{EU\text{-}NTN}$). Overall the symptoms exhibited were more severe in year two than year one.

Upon harvest of tuber progeny from the field trial, only one tuber from cultivar Pentland Crown displayed a single small lesion. After the three months storage period, tuber progeny from only Pentland Crown (maximum PTNRD DI score. 0.71 per plant) and Pentland Ivory (maximum PTNRD DI score of 0.33 per plant) displayed symptoms. The majority of Nadine tubers failed to generate a plant with the exception of tubers infected with the $PVY^{N\text{-}Wilga}$ isolate. Indeed, tubers infected with $PVY^{N\text{-}Wilga}$ produced no symptoms at harvest or after storage. Due to problems with late blight outbreak that affected

the majority of plants in this trial, it was decided to discontinue the field component of this PTNRD trial for year three.

In the PTNRD trial in controlled conditions (i.e. glasshouse grown plants) in year three:

- Only one tuber of Nadine was viable for planting; this tuber surprisingly produced symptomless progeny tubers on harvest, after three months storage all tubers developed severe PTNRD.
- The general trend of PTNRD DI in year three was very much comparable to year two.
- The overall PTNRD DI scored in year three were slightly less pronounced at harvest than year 2.
- Maris Bard, Duke of York and Pentland Ivory produced mild symptoms at harvest (PTNRD DI maximum of 0.16, 0.08 and 0.2 respectively). Pentland Crown was symptomless at harvest.
- After storage, Maris Bard tubers were the most symptomatic with a PTNRD maximum disease index of 1.17, 0.2 for Duke of York, 0.34 for Pentland Ivory; while Pentland Crown was the second most severely affected tuber progeny with a PTNRD DI maximum value of 0.9.
- The PVY isolate that elicited the most severe PTNRD was isolate 10088 (molecular group $PVY^{EU\text{-}NTN}$ strain group PVY^{E}) while the

mildest was isolate 10057 (molecular group PVYEU-NTN strain group PVY^N).

- A Kruskal-Wallis non-parametric statistical test suggested that there was a significant difference between cultivars (Chi-squared = 18.497, $p<0.001$)
- A Kruskal-Wallis non-parametric statistical test suggested that there was no significant difference between isolates (Chi-squared = 6.984, $p = 0.222$)

Over the entire three year period:

- King Edward and Maris Piper failed to produce any PTNRD symptoms.
- Nadine was the most susceptible to PTNRD. The impact of PTNRD in Nadine led to an almost complete failure to yield viable progeny from infected tubers.
- The second most severely affected cultivar was Pentland Crown.
- Symptoms in the cultivars tested were generally more severe both in years two and three, probably as a consequence of a higher level of virus titre in secondary infections than in year one (primary infection), with some PTNRD lesions being present at harvest.

- There was no obvious relationship between the PVY isolate molecular type and PTNRD severity, however in all three years, isolate 10057 (PVY^{EU-NTN}) evoked consistently the lowest symptomatology.
- The results also suggest that under field conditions in Scotland PTNRD is not observed in the cultivars tested. However, PTNRD can be triggered through storage in permissive conditions at 20°C for the cultivars Pentland Ivory and Pentland Crown.

3.3.7 Assessment of PTNRD of commercially relevant cultivars

In order to ascertain which commonly grown varieties in Scotland are potentially susceptible to PTNRD, a broad range of cultivars representing the Top 16 varieties grown in 2010, were assessed as previously described. As before, four representative PVY isolates were selected from different molecular clades (DV71 PVY^{O}, 9552 PVY^{EU-N}, DV69 PVY^{NA-NTN} and DV76 PVY^{EU-NTN}) found in Scotland. Further information relating to their relative susceptibility to PVY^{O} was identified from the European Cultivated potato Database (http://www.europotato.org/menu.php) with the view to discriminate between intrinsic PVY resistance to infection and inherent PTNRD susceptibility of selected cultivars.

Table 3.6a Assessment of PTNRD symptoms in a range of commercially relevant cultivars. Index as described in section 3.2.6. The results are an average of all four replicates for each isolate/ cultivar combination. Year/Conditions (glasshouse grown plants *i.e.* permissive)

	PVY Isolate	PTNRD index*	
Cultivar	Biotype / Molecular group / Isolate code	No storage	3 months storage
Cara	N / EU-N / 9552	0	1.52
Cara	N / EU-NTN / DV76	0	0.57
Cara	N / NA-NTN / DV69	0	0
Cara	O / DV71	0	0
Estima	N / EU-N / 9552	0.15	0.74
Estima	N / EU-NTN / DV76	0	0.93
Estima	N / NA-NTN / DV69	0.1	0.27
Estima	O / DV71	0	0.66
Hermes	N / EU-N / 9552	0.59	1.78
Hermes	N / EU-NTN / DV76	0	1.53
Hermes	N / NA-NTN / DV69	0.77	1.91
Hermes	O / DV71	0	0
King Edward	N / EU-N / 9552	0	0
King Edward	N / EU-NTN / DV76	0	0
King Edward	N / NA-NTN / DV69	0	0
King Edward	O / DV71	0	0
Lady Rosetta	N / EU-N / 9552	0	0
Lady Rosetta	N / EU-NTN / DV76	0	0
Lady Rosetta	N / NA-NTN / DV69	0	0
Lady Rosetta	O / DV71	0	0
Marfona	N / EU-N / 9552	0.47	1.6
Marfona	N / EU-NTN / DV76	0.04	0.73
Marfona	N / NA-NTN / DV69	0.08	0.4
Marfona	O / DV71	0	0.17
Maris Bard	N / EU-N / 9552	0	1.41
Maris Bard	N / EU-NTN / DV76	0	1.5
Maris Bard	N / NA-NTN / DV69	0.05	0.75
Maris Bard	O / DV71	0	1.27
Maris Peer	N / EU-N / 9552	0.21	1
Maris Peer	N / EU-NTN / DV76	0	0.19
Maris Peer	N / NA-NTN / DV69	0.57	1.29
Maris Peer	O / DV71	0	0.13
Maris Piper	N / EU-N / 9552	0	0
Maris Piper	N / EU-NTN / DV76	0	0
Maris Piper	N / NA-NTN / DV69	0	0
Maris Piper	O / DV71	0	0

Table 3.6b Assessment of PTNRD symptoms (continued)

	PVY Isolate	PTNRD index*	
Cultivar	Biotype / Molecular group / Isolate code	No storage	3 months storage
Markies	N / EU-N / 9552	0	1.86
Markies	N / EU-NTN / DV76	0	0.68
Markies	N / NA-NTN / DV69	0	0.16
Markies	O / DV71	0	0.83
Melody	N / EU-N / 9552	0.11	0.5
Melody	N / EU-NTN / DV76	0	0.5
Melody	N / NA-NTN / DV69	0	0
Melody	O / DV71	0.04	0.38
Nadine	N / EU-N / 9552	0.6	1.4
Nadine	N / EU-NTN / DV76	0	0.58
Nadine	N / NA-NTN / DV69	0.56	3.25
Nadine	O / DV71	0.17	0.13
Pentland Dell	N / EU-N / 9552	0	0.293
Pentland Dell	N / EU-NTN / DV76	0.42	0.79
Pentland Dell	N / NA-NTN / DV69	0.13	0.5
Pentland Dell	O / DV71	0	0
Picasso	N / EU-N / 9552	0	0.5
Picasso	N / EU-NTN / DV76	0	0.05
Picasso	N / NA-NTN / DV69	0	0.33
Picasso	O / DV71	0	0
Saxon	N / EU-N / 9552	0	0.04
Saxon	N / EU-NTN / DV76	0	0
Saxon	N / NA-NTN / DV69	0	0
Saxon	O / DV71	0	0
Valor	N / EU-N / 9552	0	0
Valor	N / EU-NTN / DV76	0	0.93
Valor	N / NA-NTN / DV69	0.11	2.21
Valor	O / DV71	0	0

Infected plants were grown both in controlled (glasshouse) conditions and in the field. Young leaves were inoculated *in situ*. For the field trial, only one tuber displayed any symptoms either at harvest or after storage from the cultivar Hermes, which is why the results have not been included in the table 3.6. At 21 dpi none of the field-grown tested cultivars tested positive for PVY by DAS-ELISA despite being inoculated in the same conditions as the

glasshouse trial with the same titre of inoculum. In spite of the poor infectivity of PVY isolates in field conditions, only one tuber from cultivar Hermes was found to display PTNRD, after 3 months storage. The lack of consistent infection and replication does not permit conclusions to be drawn from this field trial, which only suggest that, as reported elsewhere (Badarau *et al.*, 2010) that cultivar Hermes while displaying resistance to PVY appears to be highly susceptible to PTNRD.

Overall, for the PTNRD assessment trial in glasshouse conditions, most varieties did display PTNRD symptoms with the exception of King Edward, Lady Rosetta and Maris Piper. Cultivar Saxon displayed weak symptomatology with only one affected tuber (PTNRD DI = 1). Picasso and Melody displayed only very mild symptoms with a maximum PTNRD DI of 0.5 for isolate 9552. Cultivars displaying mild PTNRD included Estima, Pentland Dell and Valor (maximum PTNRD DI ranging from 0.5 - 1). The other varieties tested displayed moderate PTNRD, with a maximum PTNRD DI of 1 or above. This included cultivars Cara, Hermes, Nadine, Marfona, Maris Bard, Maris Peer and Markies. Results are summarised in Table 3.7. Overall for the PVY isolates tested, the isolate 9552 (molecular group $PVY^{EU-N)}$ produced the highest PTNRD DI, followed by isolate DV76 (molecular group $PVY^{NA-NTN)}$, isolate DV76 (PVY^{EU-NTN}) and isolate DV71 ($PVY^{O)}$. Using a Kruskal – Wallis test there was a statistically significant difference

between isolates (chi squared =18.534) and between cultivars (chi squared = 96.323) both with a p-value of <0.001.

Table 3.7 PTNRD index in relation to published Disease Resistance Index to PVY^{O}. Very Mild PTNRD is associated with varieties with a maximum PTNRD index less than 0.5, Mild from 0.6-1 and Moderate for all cultivars with a maximum score greater than 1.

	Resistance to PVY^{O}		
PTNRD symptoms	**Low**	**Medium**	**High**
No Symptoms (PTNRD DI=0)	King Edward, Maris Piper	Lady Rosetta	
Very Mild (PTNRD DI < 0.5)		Saxon	Picasso, Melody
Mild (PTNRD DI = 0.6 - 1)	Estima, Valor	Pentland dell	
Moderate (PTNRD DI > 1)	Maris Peer	Marfona, Maris Bard	Cara, Hermes, Nadine, Markies

The difference in PTNRD severity in Nadine between experiments (section 3.3.6 and section 3.3.7) can be explained by the fact that the Nadine plants grown for the first experiment were from micropropagated plants infected at an early stage of growth. In the latter experiment, Nadine tubers were obtained after 2 field generations beyond tissue culture prior to inoculation. While still high grade seed, the development and physiological status of the plant might have impacted on PVY infectiveness.

Figure 3.5a PTNRD symptoms in a range of cultivars infected with isolate 9552 PVY^{EU-N}

Figure 3.5b PTNRD symptoms in a range of cultivars infected with isolate 9552 PVY^{EU-N}

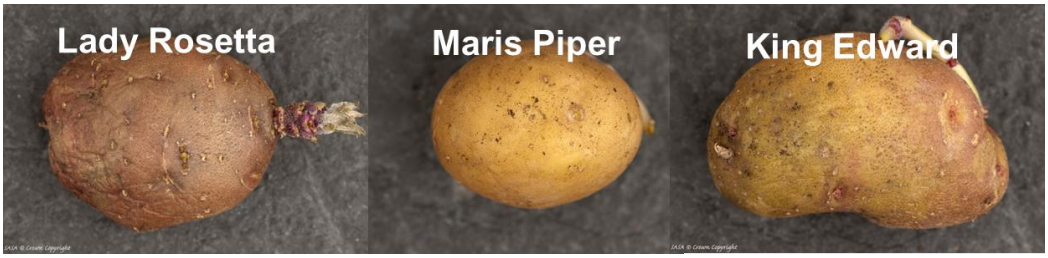


Figure 3.5c Symptomless cultivars

3.4 Discussion

With PVY^N the more prevalent strain type in Scotland and the complexity of the nomenclature for PVY, a full survey of the biological, serological and molecular characteristics were undertaken for PVY^N isolates. Chapter 2 covered the serological and molecular characterisation of isolates. This chapter focusses on the biological features of selected PVY isolates, including characterisation in a range of potato cultivars harbouring a range of PVY resistance genes, the symptomatology of cultivars infected and the susceptibility of commercially grown cultivars to developing Potato Tuber Necrotic Ringspot Disease (PTNRD), an economically important threat to the potato industry (UN Economic and Social Council, 2004). This Chapter predominantly addressed the study the pathogenicity of a selection of PVY^N field isolates in order to assess the variation between different molecular isolates of PVY in relation to the impact of PTNRD in tubers and the ability of PVY^N variants to elicit foliar mosaic symptoms. This will also allow an evaluation of the threat that different PVY isolates could potentially pose to the seed potato industry.

3.4.1 Biological characterisation of PVY isolates

Initially the biological type of PVY^N field isolates was assessed using tobacco plants and potato cultivars with known genotype and specific resistance genes (*Nc, Ny, Nz*). The ability of PVY^N isolates to cause vein necrosis (VN) in *N.*

tabacum cv. White Burley is fundamental to its derogation as a PVY^N as the N relates to tobacco necrosis (Singh *et al*., 2008). The vast majority of isolates that successfully infected tobacco (95%, n =106) produced vein necrosis. However, there were three isolates serologically identified as PVY^N that triggered mosaic instead of necrotic symptoms. The resistance specificity of differential potato cultivars harbouring PVY-specific resistance genes (*Nc*, *Ny*, *Nz*) allows the classification of PVY isolates into strain groups (Singh *et al*., 2008, Table 3.1). Six PVY^N isolates belonging to different molecular subgroups produced no observable hypersensitive response in the potato cultivars tested. This suggest that all isolates tested belong to the PVY^N strain group, with the exception of one isolate tested from the molecular $PVY^{EU\text{-}NTN}$ clade which failed to produce vein necrosis in tobacco and therefore belonging to the PVY^E strain group. In total less than 3% of all field isolates tested failed to produce vein necrosis, suggesting that the PVY^E strain represents a small minority of the Scottish PVY population. Other strains of PVY serologically typed as PVY^N but failing to produce necrosis in tobacco have been previously reported as PVY^Z strain which initiate an HR response in the cultivar Maris Bard (Chikh-Ali *et al*., 2013). The isolate L26 originally described by Hu *et al*. (2009) was biologically characterised and identified as PVY^Z by Kerlan *et al*. (2011). In this paper they also discussed another isolate HR1 which could not be confidently described as a PVY^Z due to its necrotic properties in tobacco, despite the hypersensitive reaction it elicited in the cultivar Maris Bard. Previously Blanco-Urgoiti *et al*. (1998) described isolates

as being PVY^Z on the basis that they were serologically PVY^O but cultivars containing the *Ny* gene did not initiate an HR response, thus showing that variation in biological response also occurs within PVY^O. Our study concentrated on the biological features of PVY^N due to its increasing in predominance in Scotland. Without independently testing all cultivars on both tobacco and on the standard set of potato indicator plants we are unable to rule out the possibility of the existence of PVY^Z strain in seed potatoes in Scotland. Our study demonstrates that biological strain types PVY^N and PVY^E are present within the Scottish field population and that PVY^N strains represent the majority of PVY^N isolates intercepted during field inspections.

3.4.2 Leaf symptom variation and its impact on PVY detection

Using the range of cultivars infected with the selected subset of PVY^N isolates for biological characterisation, leaf symptoms were assessed over a growing season in permissive (glasshouse-grown) conditions. Symptoms in PVY infected plants can vary depending on whether the infection is primary (from aphids) or secondary (infected progeny tuber), the virus isolate, the cultivar infected and as a result of environmental conditions (Draper *et al*, 2002). In the field it is accepted that virus symptoms are usually observed in infected progeny plants rather than during primary infection. However, in this experiment because all plants originated from *in vitro* grown culture, this enabled infection to occur at an immature stage of development and at 18 dpi symptoms were observable for most plants. DAS-ELISA was undertaken at 21

dpi to assess virus titre. The cultivars King Edward, Duke of York and Maris Bard supported relatively high virus titres and developed severe mosaic symptoms, thus implying that symptom severity can correlate in some cases to virus titre. This was as well exemplified by a low virus titre in Pentland Ivory correlating with a weak symptomatology and milder mosaic and mottling symptoms than in other cultivars. The cultivar Pentland Crown exemplified that in some cases mild symptoms in leaves can be observed despite a high virus titre, suggesting that latent PVY^{N} infections are likely to occur in this cultivar.

The PVY^{N} isolates which were selected induced a range of foliar symptoms on the cultivars tested suggesting differential aggressiveness. The PVY isolate 10088 (biotype E / molecular type EU-NTN) demonstrated that in spite of a relatively low titre at 21 d.p.i elicited severe mosaic symptoms. Of all the PVY^{N} isolates tested, the isolates belonging to the $PVY^{EU\text{-}NTN}$ molecular group triggered the most severe symptoms. In our experimental conditions, leaf symptom severity was assessed on mechanically inoculated plants grown in glasshouse conditions, representing highly permissive non-natural conditions of PVY infection and symptom development. Future experiments aiming at assessing PVY pathogenicity with respect to foliar symptoms elicited by PVY variants should be investigated in aphid-transmitted infection in field conditions.

3.4.3 Evaluation of Potato Tuber Necrotic Ringspot Disease (PTNRD) development

The initiation of PTNRD is largely dependent on the nature of PVY isolates, potato host genotype and specific environmental conditions during storage. Previous studies have identified a high degree of varietal variability in PTNRD development (Badarau *et al.,* 2010). Le Romancer and Nedellec (1997) tested 33 varieties, of these 29 were susceptible to PVY infection, 26 developed tuber necrosis, while three were tolerant to PVY and remained symptomless. They also stated that several of the cultivars tested had not yet been reported to produce PTNRD under field conditions.

Two separate experiments were undertaken to assess PTNRD symptom development. Initially a PTNRD Disease Index was assessed using potato cultivars used previously for biological characterisation of leaf symptoms. We assessed the potential association between PVY^{N} isolates and the propensity of five potato cultivars with known genetic backgrounds to develop PTNRD. In order to evaluate a relationship between leaf and tuber symptoms, leaf symptom severity and concomitantly PTNRD elicitation were assessed. These cultivars, along with Maris Piper and Nadine were assessed over a number of generations to establish whether successive generations of tuber-propagated PVY impacted on PTNRD severity. For a second study the top 16 varieties grown in Scotland were selected for PTNRD assessment in order to establish if PTRND could pose a risk in Scottish seed potato cultivars.

In the first study (results Table 3.5), initial inspection of tubers immediately at post-harvest in Year 1 demonstrated that there were no symptoms observed with the exception of cultivar Nadine which is highly sensitive to the disease as isolate 9561 (NA-NTN) produced up to 100% coverage of tubers with necrotic symptoms. For the second generation in excess of 90% of all the infected Nadine tubers planted failed to produce a viable plant. This suggests that the effect of PTNRD in Nadine, and potentially other highly sensitive cultivars, is so severe that they result in non-viable tubers. Therefore, tubers severely affected by PTRND impact negatively on tuber viability and act as a strong counter selection process by inhibiting further propagation of PVY. As a result of this, in highly susceptible cultivars, it can be assumed that PTNRD can be detrimental to the propagation of PVY. The exception to the severe necrosis in cultivar Nadine was only observed with the $PVY^{N\text{-}Wilga}$ isolate, where milder symptoms were observed. The reason for this apparent lack / low PTNRD DI for this isolate in the highly susceptible cultivar Nadine is unclear. Previous work has also associated $PVY^{N\text{-}Wilga}$ with an inability to produce PTNRD in susceptible cultivars (Kerlan *et al*., 2001, Chrzanowska *et al*., 1991). This finding has however been contradicted by studies involving North American isolates, where 81% of $PVY^{N\text{-}wilga}$ isolates tested were able to induce PTNRD. Further studies on the systemic movement of Scottish $PVY^{N\text{-}Wilga}$ strains and their relative titre in tubers might elucidate the mechanism responsible for the low levels of PTNRD produced. Alternatively if Scottish

$PVY^{N\text{-}Wilga}$ variants are unable to initiate PTNRD, the analysis of the viral genome of these isolates could reveal the molecular determinants involved in PTNRD initiation.

In all the cultivars tested over a three year period, PTNRD symptoms observed were more severe after 3 months storage at 20°C. This demonstrates that tuber storage conditions are critical to the development of PTNRD. Indeed Le Romancer and Nedellec (1997) were able to suppress PTRND development through storage at 10°C compared to tubers stored at 20°C.

PTNRD was observed at harvest in years two and three only. There was no noticeable difference in PTNRD DI between years two and three, which suggests that secondary infections will produce a comparable higher level of PTNRD DI than primary infection. Statistical analysis of year 3 of tubers after storage suggests that there is a significant difference in PTNRD expression between cultivars but not between PVY^{N} isolates.

A range of cultivars were selected from the Top 30 varieties grown in Scotland to assess the risk and impact of PTNRD to the Scottish potato industry. In glasshouse conditions, most of the cultivars selected were readily infected by PVY and produced PTNRD symptoms under favourable storage conditions (20°C). The only cultivars that failed to develop PTNRD were King Edward, Lady Rosetta and Maris Piper which supported PVY accumulation without developing any PTNRD. Overall there was a statistically significant difference between different isolates and different cultivars in PTNRD

expression. It was found in our trials that PVY^{EU-N} and the PVY^{NA-NTN} molecular types produced higher PTNRD disease index than PVY^{EU-NTN}. This suggests that all PVY^{N} isolates are capable of producing PTNRD given the right environmental conditions and susceptible cultivars (Gray *et al.*, 2010). Several factors are important in the development of PTNRD including climatic conditions, genetic characteristics of the cultivar and the genome characteristics of the virus isolate. We investigated a potential association between PVY^{O} resistance and PTNRD symptoms. Both cultivars Hermes and Nadine are classified as highly resistant to PVY^{O} (the European Potato Database www.europotato.org) and display strong resistance to infection to PVY^{O} (and PVY^{N}, SASA unpublished data) while being highly susceptible to PTNRD. Maris Piper however is highly susceptible to PVY^{O} but does not develop PTNRD. It was therefore possible that resistance to PVY^{O} in cultivars was in some way linked to PTNRD development on exposure to PVY^{N}.

Table 3.7 shows the results from the comparison of PVY^{O} resistance to PTNRD development in our glasshouse trial. No obvious relationship was found as Maris Peer displayed moderate symptoms despite being susceptible to PVY^{O}. There was however a tendency for highly PVY^{O} resistant cultivars (Cara, Hermes, Nadine, Markies) to display tuber progeny with more severe PTNRD symptoms and a potential link between PVY^{O} resistance and PTNRD could be investigated further.

Occurrence of PTNRD was assessed in a range of cultivars infected by selected PVY isolates in field conditions. The results from this demonstrate that under field conditions in Scotland PTNRD does not readily develop in primary infected plants and that virus acquisition is slower and less likely in the field even with a high level of inoculation. As a consequence, PTNRD development is significantly reduced resulting in a lack of observable PTNRD in field conditions. Due to the exceptionally wet summer when this experiment was undertaken, it was particularly challenging to generate significant infection levels in a number of biological replicates to obtain meaningful results. Further studies using glasshouse-inoculated plants and further shifted outdoors until harvest might be the preferable option to obtain reproducible data between years.

3.4.4 Relationship between leaf symptomatology and PTNRD

The work carried out in this chapter highlights the differences between leaves and tuber symptoms in cultivar / PVY isolate combinations. It has previously been stated that there is a poor relationship between foliar symptoms and those seen on tubers (Halterman *et al.*, 2012). From our (preliminary) study, we can conclude that no obvious relationships were found between leaf symptom severity and PTNRD. Of the cultivars assessed for biological characterisation Pentland Crown produced the least symptomatic leaves and conversely the most symptomatic tubers. In contrast the cultivar King Edward displayed highly symptomatic leaves however failed to produce PTNRD in tuber

progeny. In cultivar Maris Piper the leaf symptoms (although not recorded for this chapter) were not as severe as those displayed by Kind Edward but in this case as well, no tubers displayed symptoms of PTNRD. In cultivar Nadine, leaves on infected plants became highly necrotic as if displaying a hypersensitive reaction towards PVY with symptoms similar to those demonstrated by Kerlan *et al*. (2011) for cultivar Maris Bard in response to infection with PVY^{Z}. In Nadine, most leaves dropped from the plant, (as observed for PVY^{O}) suggesting that the plant is extremely sensitive to PVY infection and perhaps displays the onset of necrotisation as a reaction to PVY infection, which could be investigated further. The only PVY isolate that did not elicit a necrotic reaction in either the leaves or tubers of Nadine was $PVY^{N\text{-}Wilga}$. The lack of symptom development in either leaves or tubers by $PVY^{N\text{-}Wilga}$ has been reported previously by Kerlan *et al*. (2001) and Glais *et al.* (2005), further work aimed at investigating the molecular characteristics of $PVY^{N\text{-}Wilga}$ could help to identify the necrotic determinants for both leaves and tubers.

3.4.5 Implications of PVY^{N} pathogenicity to the potato industry in Scotland

Scotland has a low incidence of virus in potato crops. The seed certification scheme in Scotland relies on the visual inspection of growing crops and sets strict tolerances towards observable virus symptoms of mosaic and leafroll for seed crops, these tolerances are dependent on both the mosaic severity and the

seed grade. Therefore, interception of PVY is achieved on field observation of mosaic symptoms. Inspectors are trained annually for the recognition of the most frequently grown potato cultivars and on foliar virus symptoms. It is generally accepted that PVY^N variants elicit milder symptoms than PVY^O (Widemann *et al*., 1988) therefore, a significant proportion of PVY^N infected plants might result in latent infection (*i.e.* symptomless) and could be missed during inspection of growing crops and roguing of potato crops; *i.e.* the removal of any plants displaying viral symptoms would be limited to the plants displaying obvious mosaic symptoms and consequently selecting PVY variants that elicit milder symptoms. This could potentially explain why PVY^N is becoming more prevalent as opposed to PVY^O (Ragsdale *et al*., 2001).

With leaf symptoms being the main factor used to detect virus presence in seed crops in Scotland, it was therefore relevant to study PVY^N pathogenicity with respect to leaf symptom severity in a selection of cultivars with different resistance characteristics. The cultivar Pentland Crown had a propensity to develop milder symptoms despite supporting a comparable or higher infection titre than other cultivars. Pentland Crown is not a commonly grown variety in Scotland (failed to reach the top 30 varieties and in 2011 as only 3.5 hectares of Pentland Crown was commercially grown in Scotland) (SASA, Seed Potato Certification data). However, the finding that this cultivar produced nil to very mild symptoms despite supporting a relatively high virus titre indicate that not all cultivars can be assessed accurately for virus infection based solely on leaf

symptoms observation. Such cultivars should be considered for different virus management strategies, such as enforcing compulsory post-harvest virus testing on high seed grade to ensure cleanliness of stocks in early field generations. This would prevent propagation of secondary infection by downgrading/eliminating crops that are outside the set tolerances. While, for the majority of cultivars visual inspection is valid, it is worth considering testing new cultivars, and those with increasing popularity for leaf symptomatology in relation to virus titre to ensure that virus field inspections are suitable for the certification of these varieties. The assessment of leaf symptomatology revealed inherent variation in symptom development between a range of cultivars and PVY isolates combinations, consequently affecting the detection of mosaic symptoms during field inspections. Gray *et al.* (2010) warn that limited testing for virus susceptibility and the use of visual inspection schemes have led to symptomless carriers of PVY. Our data demonstrates that visual inspection might underestimate the prevalence of PVY^{N}, and depending which cultivar are infected latent infection might develop which will result in a unreported source of PVY inoculum in the example of Pentland Crown . The fact that the most common molecular type $PVY^{EU\text{-}NTN}$ elicits the strongest symptoms (and assuming that foliar symptoms might be comparable in glasshouse and field conditions), suggests that increase in PVY^{N} might not only be a direct consequence of milder symptomatology and failed interception during inspections of growing crops.

Consequently, other factors are likely to be responsible for PVY^N prevalence. These will be discussed in Chapter 4

Variations in symptom severity were observed for some cultivars such as Maris Bard and King Edward across a growing season. For Maris Bard, a continual increase in symptom severity was observed during the monitoring period while, contrastingly, symptoms in Duke of York were relatively constant. This demonstrates that even in symptomatic cultivars the timing of inspection could have an impact on the grading of an inspected crop and that field symptoms may differ significantly at different time. Importantly crops are inspected twice during the growing season to circumvent this potential drawback.

In order to assess the threat of PTNRD development in our environmental conditions, PVY pathogenicity in relation to PTNRD development was assessed. This demonstrated that, while PTNRD is rarely observed in field conditions in Scotland, PTNRD can be elicited in permissive conditions (*i.e.* glasshouse grown plants). This suggests in accordance with Le Romancer and Nedellic (1997) that environmental conditions may play a large role in the development of the disease. Recently Hutton *et al*. (2013) reported the first incidence of PTNRD in Ireland, again the suppression of the disease here may be linked to climatic conditions as the lesions appeared only after storage for a month in ambient conditions. Such assessment should be considered when it

comes to choosing varieties suitable for export to countries with more favourable environmental conditions for PTNRD development. Due to the method of inspections and the risk of PTNRD to exported seed, potato breeders may consider the benefits of PTNRD resistant cultivars and particularly those that are highly leaf symptomatic. Integrated with an effective roguing regime, cultivars which are highly leaf symptomatic but asymptomatic in tubers (e.g. King Edward) may be beneficial in a classification scheme that relies on the visual detection of virus symptoms in a growing season.

3.5 Conclusions

The biological characterisation of a sub-set of PVY isolates showed that a large majority of those found in Scotland which belong to the PVY^N serotype can be classified within the PVY^N strain group. Only one isolate could be identified as belonging to the PVY^E strain group which suggest that a small proportion of PVY found in Scotland fall within this strain group.

Overall, pathogenicity tests revealed strong variability in symptoms development in both leaves and tubers for different potato cultivar and PVY isolates combinations. In total five cultivars were assessed for leaf symptoms. Of the cultivars tested three cultivars displayed severe leaf symptoms (Maris Bard, King Edward and Duke of York). Pentland Ivory produced milder symptoms, but this was associated with lower virus titre. In the cultivar Pentland Crown symptoms were less pronounced and in some cases could be considered as asymptomatic. The results demonstrate that cultivars such as Pentland Crown support latent PVY infection and therefore present a high risk of PVY propagation and undetectable source of PVY inoculum.

Sixteen of the nineteen cultivars tested in our surveys produced PTNRD in permissive conditions (glasshouse grown and controlled storage temperature). However, in a field trial it proved difficult to initiate symptoms in any cultivar. King Edward, Maris Piper and Lady Rosetta failed to produce any tuber symptoms with the isolates tested in either glasshouse or field conditions

demonstrating that these cultivars are tolerant for PVY and display strong resistance to PTNRD. The deduction from this is that PTNRD can affect a wide range of cultivars grown throughout the world if grown in permissive conditions. There is a potential threat of PTNRD from Scottish PVY^{N} isolates in locally grown potato cultivars. At present PTNRD has not been reported from Scottish seed potatoes and there are two potential reasons for this: (*i*) misdiagnosis of symptoms as PTNRD can resemble other tuber disease symptoms such as spraing, (*ii*) climatic conditions that do not support the development of PTNRD development in tubers.

In relation to pathogenicity studies for foliar and tuber symptom development, results suggest the absence of a clear relationship between symptom expression in leaves and PTNRD in tubers, either for each PVY isolate tested on a given cultivar and as well for a given PVY isolate on a range of cultivars. Therefore, predictions of PTNRD tuber symptoms cannot be made from observation of leaf symptom severity. This would also suggest that different molecular determinants in the PVY genome are likely to be involved in eliciting necrosis in tobacco to those producing strong foliar symptoms in potato and those that relate to the development and PTNRD in tubers.

Chapter 4 - Transmission and distribution of PVY variants in field conditions

4.1. Introduction

4.1.1 Aphid transmission of PVY

Viruses such as PVY can be transmitted either horizontally plant to plant (aphid-borne infection from neighbouring infected plants termed primary infection) or vertically plant to tuber (tuber-borne infection from an infected plant termed secondary infection) (Hull, 2001). While it is widely accepted that the main cause of seed health degeneration from PVY is through tuber-borne infections (Whitworth *et al.*, 2012). PVY must be acquired first through infection by a viruliferous aphid, therefore efficient management of PVY relies on a better understanding of PVY epidemiology and in particular the effectiveness of viral acquisition and deposition (Boquel *et al.*, 2011). PVY is transmitted in a non-persistent manner requiring only a brief acquisition and inoculation period (Pirone and Harris, 1977). Some aphid species are able to transmit the PVY effectively, while others are not. In total around 40 species are known to be a vector of PVY (Edwardson and Christie, 1997, Quenouille *et al*, 2013). However, there is significant variation in their transmission efficiency (Sigvald, 2008). *Myzus persicae* (Peach potato aphid) is known to be the most effective transmitter of PVY and as a result is given a relative transmission efficiency factor (REF) of 1 (Verbeek *et al.*, 2010). All other aphids are measured against this species in laboratory trials and are therefore

given a REF value to *Myzus persicae*, which will be a fraction of 1. Vector pressure of aphids can be calculated through multiplication of each aphid species' REF value by the number caught (Verbeek *et al*., 2010).

In order to investigate what are the key factors driving the PVY population shift from PVY^{O} to PVY^{N} serotypes; the parameters that control $PVY^{EU\text{-}NTN}$, $PVY^{NA\text{-}NTN}$ and PVY^{O} transmission and distribution in field conditions were investigated. Analysis of the timing of virus transmission in conjunction with aphid vector pressure should give a good indication as to whether different species are transmitting different strains of PVY. Aphid species composition changes during the season (Kirchner *et al*, 2013), with different species contributing to the overall vector pressure this would mean that should a change in the pattern of transmission occur between different isolates this may indicate that different species are responsible for transmitting different isolates.

4.1.2 Timing, frequency and distribution of PVY isolates.

The previous experimental set-up on the timing of transmission of PVY^{O} and PVY^{N} serotypes (Pickup *et al*., 2009) has been extended to assess the behavior of the 3 selected PVY isolates DV76 $PVY^{EU\text{-}NTN}$, DV69 $PVY^{NA\text{-}NTN}$ and DV71 PVY^{O}. In these trials, aphid species and virus transmission were monitored weekly, giving the possibility to link spatio-temporal analysis of virus distribution, transmission to tobacco bait plants and the concomitant

occurrence of aphids using local suction trap data. Van Hoof (1977) showed that the use of tobacco plants along with suction trap data was an effective method to monitor infection pressure within a crop.

The incidence and distribution of PVY isolates in bait potatoes was assessed in order to look at the effectiveness of secondary transmission to tubers. The distance over which PVY strains are transmitted could also be relevant, as the further the distance PVY can spread from the initial source of infection the higher the incidence of primary infections in nearby crops. The movement of the virus through the plant is an important factor, as the faster a virus can spread systemically to invade the whole foliage of a potato plant the more likely an aphid can acquire and transmit it to another plant. Potato tubers are grown over several generations, so the ability of the virus to infect the whole plant including tubers is of critical importance to its survival and spread. Tubers infected with virus, when planted will produce a virus infected plant (secondary infection) which will generate further inoculum source (Rahman and Akanda, 2008). The comparison of transmission range and frequency for each isolate will help to identify contributing factors in the selection and fitness of specific PVY species and explain the nature of the PVY population structure.

4.1.3 Within plant competition

When multiple viruses or virus strains of the same virus species infect a plant, competition can occur within the plant (Power, 1996, Syller, 2012), conditioning the success of one virus/strain over the other. In order to assess whether within plant competition contribute significantly to the selection of specific PVY strains, we assessed in controlled conditions whether infection of a combination of PVY species $PVY^{EU\text{-}NTN}$, $PVY^{NA\text{-}NTN}$ and PVY^{O} at different concentrations in individual potato plants result in the prevalence of a specific PVY species by assessing their distribution and frequency of transmission in tubers.

4.2. Methods

4.2.1. Field trial layout to assess timing of transmission and distribution of PVY isolates

The field trial plot (Pickup *et al*., 2009) consists of 450 virus-free Maris Piper tubers (Pre-basic grade 3, Jim Cruickshank, Aberdeen, UK). Twenty one tuber-borne infected potatoes were used as the inoculum source of PVY. Of these 7 plants were infected with either $PVY^{EU\text{-}NTN}$, (isolate DV76), $PVY^{NA\text{-}NTN}$ (isolate DV69) or PVY^{O} (isolate DV71) (See highlighted isolates in Figure 4.1 and plot plan Figure 4.2). Each tuber was planted approximately 10 cm under the soil. In 2010 the planting date was 29th of April, in 2011 it was the

19th of April and the 9th of May in 2012. The choice of date of planting, was weather dependant. Foliar emergence was observed at approximately 4-5 weeks post planting, at which time *Nicotiana debneyi* bait plants were exposed and replaced weekly. This continued for a period of 9 to 11 weeks, until haulm destruction. Infector plants were cv. Maris Piper with the exception of PVY^{EU-NTN} which was cv. Nadine in 2010; these were replaced by Maris Piper for 2011 and 2012 following successful infection of Maris Piper plants. As the infectors alternate throughout the plot, one of each isolate is positioned at the centre of each of the *Nicotiana debneyi* rows to minimise any positional effect on transmission. Week-to-week variation in PVY transmission was monitored by changing the *N. debneyi* bait plants each week of which there were 6 rows of 24 plants. At 3-weeks post incubation in insect-proof glasshouses, *N.debneyi* plants were tested for the presence of virus by DAS-ELISA. In 2011 samples that tested positive using ELISA were subsequently further discriminated using Reverse Transcription-PCR to differentiate between PVY^{EU-NTN} and PVY^{NA-NTN} isolates.

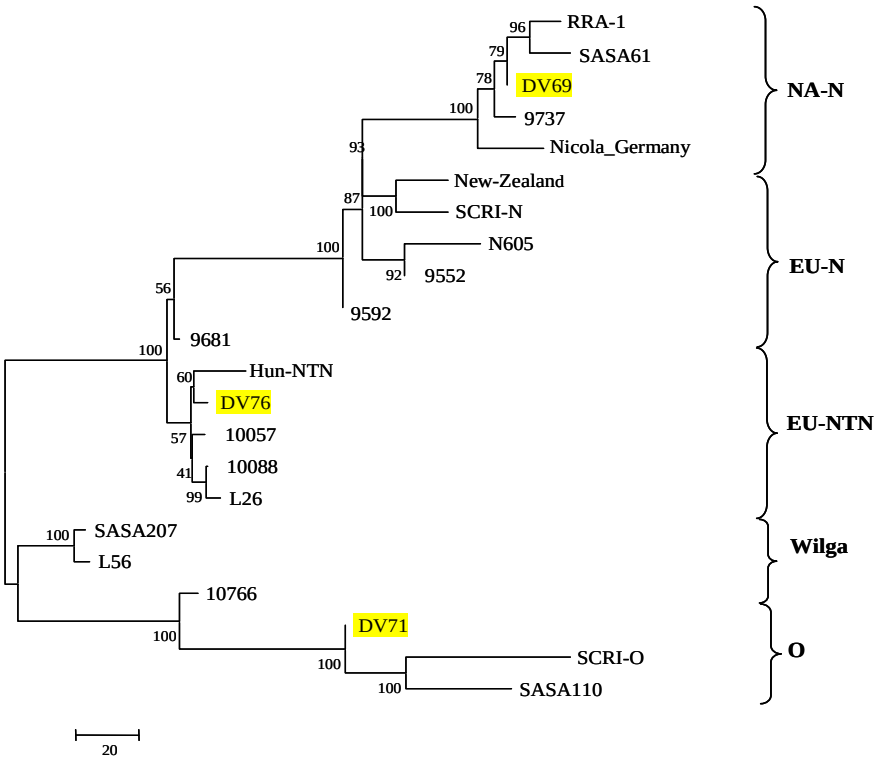
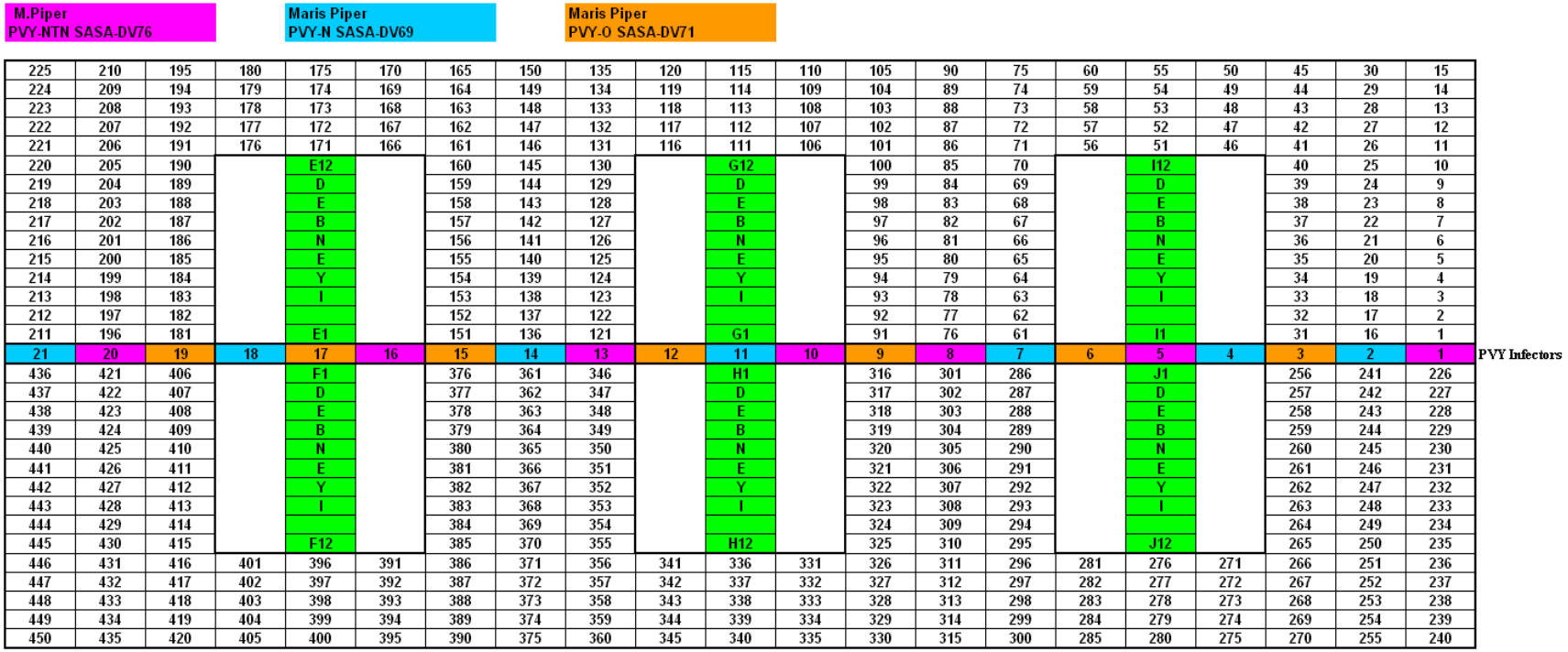


Figure 4.1. The phylogenetic relationship of the PVY isolates used in field trials (highlighted). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). Branches corresponding to partitions reproduced in less than 70% bootstrap replicates are collapsed. The evolutionary distances were computed using the number of differences method (Nei and Kumar, 2000) and are in the units of the number of amino acid differences per sequence. The coding data was translated assuming a Standard genetic code table. There were a total of 3209 amino acid positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamure *et al.*, 2011).



225	210	195	180	175	170	165	150	135	120	115	110	105	90	75	60	55	50	45	30	15
224	209	194	179	174	169	164	149	134	119	114	109	104	89	74	59	54	49	44	29	14
223	208	193	178	173	168	163	148	133	118	113	108	103	88	73	58	53	48	43	28	13
222	207	192	177	172	167	162	147	132	117	112	107	102	87	72	57	52	47	42	27	12
221	206	191	176	171	166	161	146	131	116	111	106	101	86	71	56	51	46	41	26	11
220	205	190		E12		160	145	130		G12		100	85	70		I12		40	25	10
219	204	189		D		159	144	129		D		99	84	69		D		39	24	9
218	203	188		E		158	143	128		E		98	83	68		E		38	23	8
217	202	187		B		157	142	127		B		97	82	67		B		37	22	7
216	201	186		N		156	141	126		N		96	81	66		N		36	21	6
215	200	185		E		155	140	125		E		95	80	65		E		35	20	5
214	199	184		Y		154	139	124		Y		94	79	64		Y		34	19	4
213	198	183		I		153	138	123		I		93	78	63		I		33	18	3
212	197	182				152	137	122				92	77	62				32	17	2
211	196	181		E1		151	136	121		G1		91	76	61		I1		31	16	1
21	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
436	421	406		F1		376	361	346		H1		316	301	286		J1		256	241	226
437	422	407		D		377	362	347		D		317	302	287		D		257	242	227
438	423	408		E		378	363	348		E		318	303	288		E		258	243	228
439	424	409		B		379	364	349		B		319	304	289		B		259	244	229
440	425	410		N		380	365	350		N		320	305	290		N		260	245	230
441	426	411		E		381	366	351		E		321	306	291		E		261	246	231
442	427	412		Y		382	367	352		Y		322	307	292		Y		262	247	232
443	428	413		I		383	368	353		I		323	308	293		I		263	248	233
444	429	414				384	369	354				324	309	294				264	249	234
445	430	415		F12		385	370	355		H12		325	310	295		J12		265	250	235
446	431	416	401	396	391	386	371	356	341	336	331	326	311	296	281	276	271	266	251	236
447	432	417	402	397	392	387	372	357	342	337	332	327	312	297	282	277	272	267	252	237
448	433	418	403	398	393	388	373	358	343	338	333	328	313	298	283	278	273	268	253	238
449	434	419	404	399	394	389	374	359	344	339	334	329	314	299	284	279	274	269	254	239
450	435	420	405	400	395	390	375	360	345	340	335	330	315	300	285	280	275	270	255	240

Figure 4.2: Plot layout of the epidemiology field trial. The centre line of the plot indicates the location of the different isolate infector plants. The position of the six rows of *Nicotiana debneyi* bait plants is also shown.

4.2.2. Aphid vector pressure.

Aphid monitoring for assessment of aphid vector pressure is achieved through the use of suction trap data. The Edinburgh trap is located at Latitude: 55°56.9 Longitude: -3°18.8, which is less than 1km from the field plot. The traps vary very little from a standardized design which draws air in at a rate of approximately 0.75 m^3s^{-1} through an aperture 12.2 m above ground level (Harrington, 2001). The aphid suction trap (Taylor, 1951) produces a constant vacuum, sucking airstreams down into the bottle; this catches winged aphids and other insects that are flying at this height. Each day at 10:00 British summer time a customized bottle, containing 100mL of stock solution (65% Industrial Methylated Spirit (IMS), 5% glycerol and 30% water) is replaced. Aphids are identified by the entomology team at SASA and daily catches are recorded on to a databasse. Aphid vector pressure is calculated by multiplying the number of aphids caught in a given week by the vector efficiency (Table 4.1).

Figure 4.3: Aphid Suction Trap, located in Gogarbank, Edinburgh

Table 4.1: Relative efficiency factors of aphid vector species known to transmit PVY. (SASA and Food and Environment Research Agency based on Sigvald (1984) and DeBoxk and Piron (1990)).

Aphid Species	Relative Efficiency Factor (REF)
Acyrthosiphon pisum	**0.7**
Aphis fabae	**0.1**
Brachycaudis helichrysi	**0.21**
Brevicoryne brassicae	**0.01**
Hyperomyzus lactucae	**0.16**
Macrosiphum euphorbiae	**0.2**
Metopolophium dirhodum	**0.3**
Myzus ornatus	**0.2**
Myzus persicae	**1**
Sitobion avenae	**0.01**

4.2.3. Weekly transmission to tobacco *N. debneyi* plants

To assess the timing and frequency of transmission throughout the season, tobacco bait plants *Nicotiana debneyi* were used as these have been shown to be effective indicators of field transmission of Potato virus Y (Van Hoof, 1977). *Nicotiana debneyi* plants were grown from seeds in John Innes number two compost (Berkshire) with two plants per pot. After 6 weeks growth inside an aphid free glasshouse plants were transferred to the field and labelled according to their position within the plot. After one week exposure in the field, the plants were fumigated using Bug Clear Ultra (MAPP 12905) to ensure all aphids were killed and PVY transmission was stopped. Plants were then transferred in an aphid free glasshouse at 20^{O}C daytime and 16^{O}C night temperature at 16hr to 8hr respectively. The plants were grown for another 3

weeks to ensure PVY levels increased to a detectable level for DAS_ELISA. Leaves were then sampled and tested by DAS-ELISA as described in Chapter 2.2.2.

4.2.4. PVY diagnosis from tubers

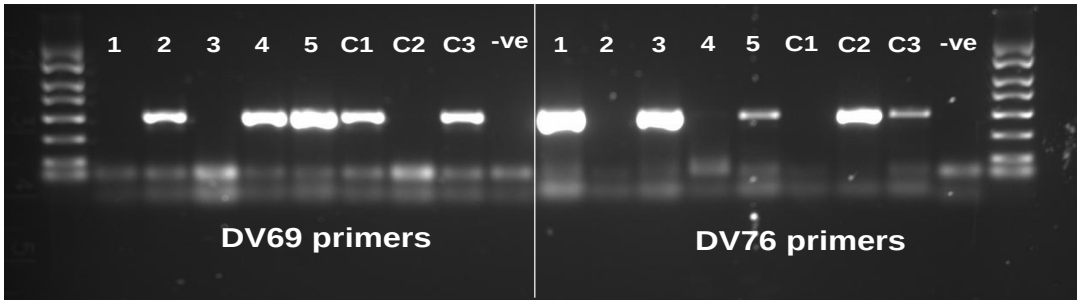
At two weeks post-haulm destruction, potato plants were harvested and tubers from each individual plant collected. Tubers were stored in dark at 5ºC for a period of 6 months. Following storage, three tubers per plant were selected and the region of tuber where sprouting occurred was cored out using a melon scoop. These eye-plug cores were then submerged in a Gibberellic acid solution (10mM) for 10 min to initiate sprouting, then placed skin down on marked 4.25'' by 5.25" crystal bags (Kenro Ltd, Swindon) to dry for 12 hours. Eye-plugs were then planted in a tray containing multipurpose compost (Bulrush, Londonderry, UK) mixed with 5% perlite (Sinclair, Gainsborough) and grown in an insect-free glasshouse (16h day at 20ºC and 8h night at 15ºC). After a period of 2-4 weeks (dependent on emergence) shoots and leaves were tested by DAS-ELISA as described in Chapter 2 using monoclonal antibodies against $PVY^{O/C}$ or PVY^{N}. For 2011 and 2012 all samples that tested PVY^{N} positive by DAS-ELISA were subsequently genotyped using Reverse Transcription (RT)-PCR to differentiate between $PVY^{NA\text{-}NTN}$ and $PVY^{EU\text{-}NTN}$ molecular types. For each plant 3 tubers were tested and the proportions of infected daughter tubers per plant were recorded.

4.2.5. Genotyping of PVY isolates

To identify the molecular group of the PVY^N isolates, total RNA was extracted from infected leaves of *N.debneyi* and potato plants using the MagExtractor-RNA Kit (Toyobo, Japan) performed on the magnetic particle processor KingFisher (Thermo Scientific, Basingstoke, UK) and RNA was stored at -20°C for future use (Mortensen *et al*, 2010). A two step RT-PCR was performed. Step one per reaction contained 5.9µL of Sigma H_2O, 10 µL of 2x Jumpstart REDTaq Ready Mix (Sigma, UK) containing 2 µL of RNA, and 0.05µL of Moloney murine leukaemia virus reverse transcriptase (Promega, Southampton UK) with 0.05µL PVY R1 reverse primers at a concentration of 100 µM. The PCR was run at 48ºC for 30 minutes then 94ºC performed on an MJ Research Peltier Thermal Cycler PTC-200 (Waltham, UK). The plate was removed from the block and 1 µL of DV 76 or DV 69 of 5 µM forward and reverse primers were added to each well to give a final volume of 20 µL, the plate was returned to the thermocycler and it was set to 95 °C for 2 mins then 32 cycles of 94 ºC for 30 seconds, 51 ºC for 30 seconds and 72 °C for 1 min for 40 cycles, then 72 °C for 7 mins. Gels were run and visualised using a GelDoc-IT TS imaging system transilluminator.

Table 4.2: Primer sequence used for the molecular genotyping of PVY^{EU-NTN} (DV76) and PVY^{NA-NTN} (DV69) isolates.

Primer	Sequence (5'-3')
DV-76, R1FWD	CTATAGAGTTGGTGGTATTCCTAAT
DV-76, R1REV	AAAACCCGCCTTGAATAGG
DV-69, R1FWD	TTATAGAGTTGGTATTCCTGGG
DV-69, R1REV	GTGAAAAACCTGCCTTAAACAAC



Sample Number	Relates To	Isolate Present
1	Plant 1 Post Harvest 2010	DV76
2	Plant 16 Post Harvest 2010	DV69
3	Plant 53 Post Harvest 2010	DV76
4	Plant 62 Post Harvest 2010	DV69
5	Plant 241 Post Harvest 2010	DV76 and DV69
C1	DV69 Control	DV69
C2	DV76 Control	DV76
C3	DV69 and DV76 Dual Infection Contol	DV76 and DV69

Figure 4.4 Example of PCR genotyping of PVY$^{EU\text{-}NTN}$ (DV76) and PVY$^{NA\text{-}NTN}$ (DV69) with corresponding samples in the adjoining table. This figure shows PCR gel results for RNA extracted from Maris Piper potato cultivars harvested in 2010. The table below relates to the sample number on the top line of the gel electrophoresis print out, and shows which isolates are present. The left picture shows where DV69 (NA-NTN) primers are used and the right panel shows where DV76 (EU-NTN) primers are used. The sequence of the primers is shown in table 4.2. The results when combining the two gel results are shown on the right hand side of the table and demonstrate that these primers are able to detect individual infection and dual infection with these virus isolates.

4.2.6. Statistical analysis of field transmission data.

The *Chi*-square (Microsoft Excel statistical package) was used to test independence of distribution of PVY strains by comparing the expected frequencies with the actual frequencies found (Fowler *et al.*, 1998) ensuring there is no association between different strains of the virus. The test was performed on both PVY^{O} with PVY^{N} and also for $PVY^{EU\text{-}NTN}$ against $PVY^{NA\text{-}NTN}$. In order to assess whether there was a significant difference between $PVY^{EU\text{-}NTN}$ and $PVY^{NA\text{-}NTN}$ infection a McNemar's test (McNemar, 1947) on matched pairs was used as they were found to be related in the *Chi*-square test. The version used to perform the test can be found here: http://faculty.vassar.edu/lowry/propcorr.html .

4.2.7 Within plant viral strain competition

A trial was set up to test the hypothesis of within plant competition between strains and assess their relative fitness (*i.e.* ability to outcompete another virus strain to ultimately detect a single virus strain) when more than one strain infects plant. Dual inoculation was carried out using defined volume and concentrations of each PVY isolate. To prepare the inoculum 6 *Nicotiana benthamiana* and 6 *Nicotiana tabacum* cv. White Burley plants were inoculated with one of the following isolates: PVY^{O} (DV71), $PVY^{EU\text{-}NTN}$ (DV76) or $PVY^{NA\text{-}NTN}$ (DV69). Inoculum sap was prepared in 50mM Sorensens phosphate buffer pH7.2. Titre of PVY inoculum for PVY^{O}, $PVY^{EU\text{-}NTN}$ or $PVY^{NA\text{-}NTN}$ was determined by DAS-ELISA using PVY polyclonal

antibodies (SASA PVY PAb coating and conjugate antibodies). Absorbance was measured at OD_{405nm} using a Sunrise microplate absorbance reader (Tecan, Mannedof, Switzerland). The concentration of all isolates was comparable on the original tests so no adjustment of inoculum concentration was required. Dilutions were then made for each inoculum, (undiluted, 2-fold and 10-fold dilution). Plants were inoculated using 3 x 50 µL of sap inoculum per isolate per plant at 2 weeks post planting of sprouted high grade Maris Piper tubers. Four replicates were used for each inoculation combination. Firstly, an equal concentration of each isolate done using full concentrated sap for both isolates. A double concentration of one isolate over the other where one isolate was full concentrated sap (2x concentration of the other isolate) and the other was diluted 1:1 with H_2O. Finally ten times concentration of one isolate over the other where the 10x isolate was a full strength concentration and the other isolate was diluted on a 1:9 ratio with H_2O. Positive controls were also inoculated for each isolate and non-inoculated plants were used as negative controls. Additionally an aphid experiment was undertaken simultaneously in order to compare results. The aphids used were *Myzus persicae* which had been starved aphids overnight starving aphids makes them more receptive to the virus (Powell, 1993). Again there were four replicates for each inoculation combination but concentration of the virus was not measured for this experiment. Ten aphids were placed in each clip cage and attached to an infected *Nicotiana* plant. The aphids were given 10 min to feed

on infected plant then moved to uninfected Maris Piper plants for 1 h, before being removed.

To test the level of infection within the progeny tubers these were eye-plug cored and submerged in a gibberellic acid solution (10 mM) for 10 min to initiate sprouting then dried overnight prior to planting. Five tuber cores were planted for each plant in a tray containing multipurpose compost (Bulrush, Londonderry, UK) mixed with 5% perlite (Sinclair, Gainsborough) and grown in glasshouse conditions of 16 hr days at 20 ^{o}C and 8 h nights at 15 ^{o}C. After a period of 2-4 weeks (dependent on emergence) shoots and leaves were bulked per plant and for any with a mixed infection of PVY^{N} serotype and a PVY^{O} serotype these were tested by DAS-ELISA using monoclonal antibodies For plants that were inoculated with $PVY^{EU\text{-}NTN}$ and $PVY^{NA\text{-}NTN}$ the isolate type present was assessed using (RT)-PCR.

4.3. Results

4.3.1. Frequency and timing of transmission of PVY isolates in 2010

Weekly transmission of PVY isolates was assessed during each season by DAS-ELISA testing of tobacco bait plants (*Nicotiana debneyi*). Week 1 of the trail is considered from the point that the majority of the crop has emerged, this is usually approximately 6 weeks after planting. DAS-ELISA using monoclonal antibodies raised against $PVY^{O/C}$ and PVY^{N} can only distinguish between PVY^{O} or PVY^{C} and PVY^{N} serotypes, $PVY^{EU\text{-}NTN}$ and $PVY^{NA\text{-}NTN}$ infected plants are both counted positive for PVY^{N}. This suggests that on the basis of ELISA testing 14 infector plants were serologically PVY^{N} and 7 were PVY^{O}. Assuming a similar timing of transmission and transmission efficiency for all the PVY isolates, a 67% - 33% ratio would be expected in the weekly detection of the virus in *N. debneyi* bait plants.

4.3.1.1. Weekly frequency of transmission

In 2010 the transmission of both PVY^{O} and PVY^{N} isolates was observed from week 4 of the trial (starting on the 21st of June 2010) where 3% and 1% of tobacco bait plants were found ELISA positive for PVY^{N} and PVY^{O} respectively. The PVY^{N} transmission rate reached a maximum frequency at week 7, 35% of *N.debneyi* plants were infected, (starting 15th of July) and week 9 (29th of July). A comparable transmission pattern was observed for

PVY^{O} with the highest transmission frequencies observed at week 7 and week 9, with 16 % and 14.5 % of *N. debneyi* plants being infected, respectively. These preliminary results show that isolates belonging to the PVY^{N} serotype ($PVY^{EU\text{-}NTN}$ and $PVY^{NA\text{-}NTN}$) and PVY^{O} are comparably transmitted across the season. Overall, the timing of transmission was similar for both PVY^{O} and PVY^{N} serotypes (Figure 4.5), with a pattern matching the overall PVY vector pressure (Figure 4.5), suggesting that PVY^{N} and PVY^{O} are likely to be transmitted by the same aphid species.

In total 293 plants from a potential 1,728 were infected with PVY. The majority of infected tobacco bait plants (73%) tested PVY^{N} positive while 27% were PVY^{O} positive. Plants could be infected by more than one strain of PVY. Any plant that was infected by both strains of the virus was counted as two positives, one for each strain.

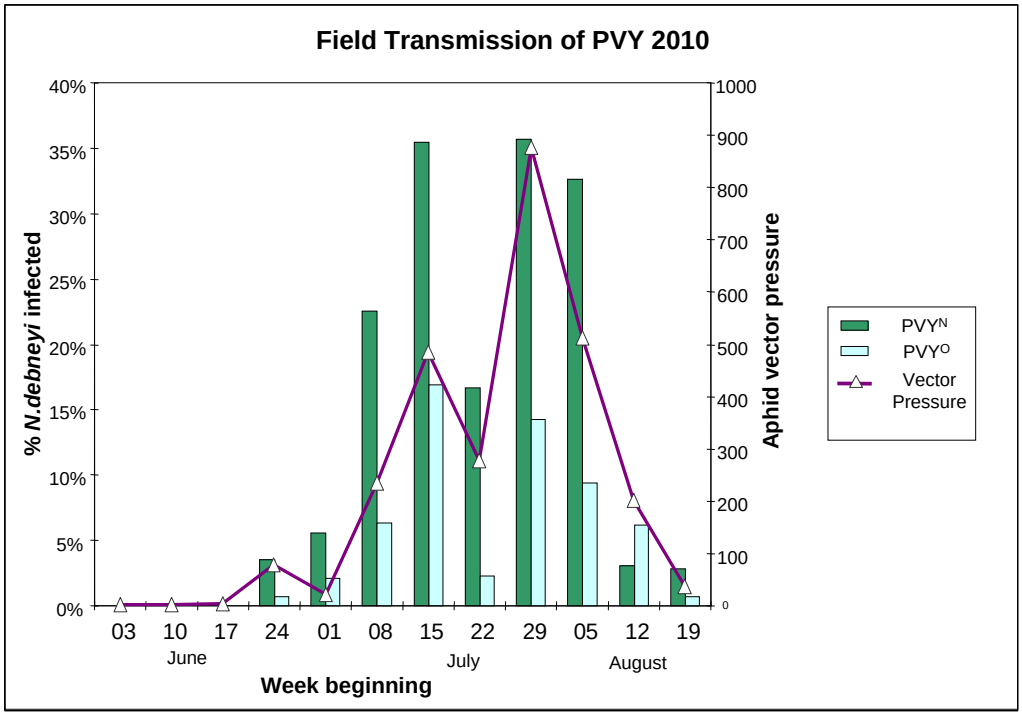


Figure 4.5 Weekly frequency of transmission of PVY^{O} and PVY^{N} serotypes (2010 season). The frequency of transmission is expressed as the percentage of ELISA-positive *Nicotiana debneyi* plants per week. The weekly PVY aphid vector pressure (Gogarbank suction trap, Edinburgh, UK) is indicated Aphid vector pressure is the relative efficiency factor (Table 4.1) of each aphid species multiplied by the total number of those species captured then added together. Therefore, as an example if only 2 *Myzus persicae* (REF=1) and 5 *Metopolophium dirhodum* (REF=0.3) were captured in one week, the vector pressure would be 3.5.

4.3.1.2. Distribution and incidence of PVY isolates

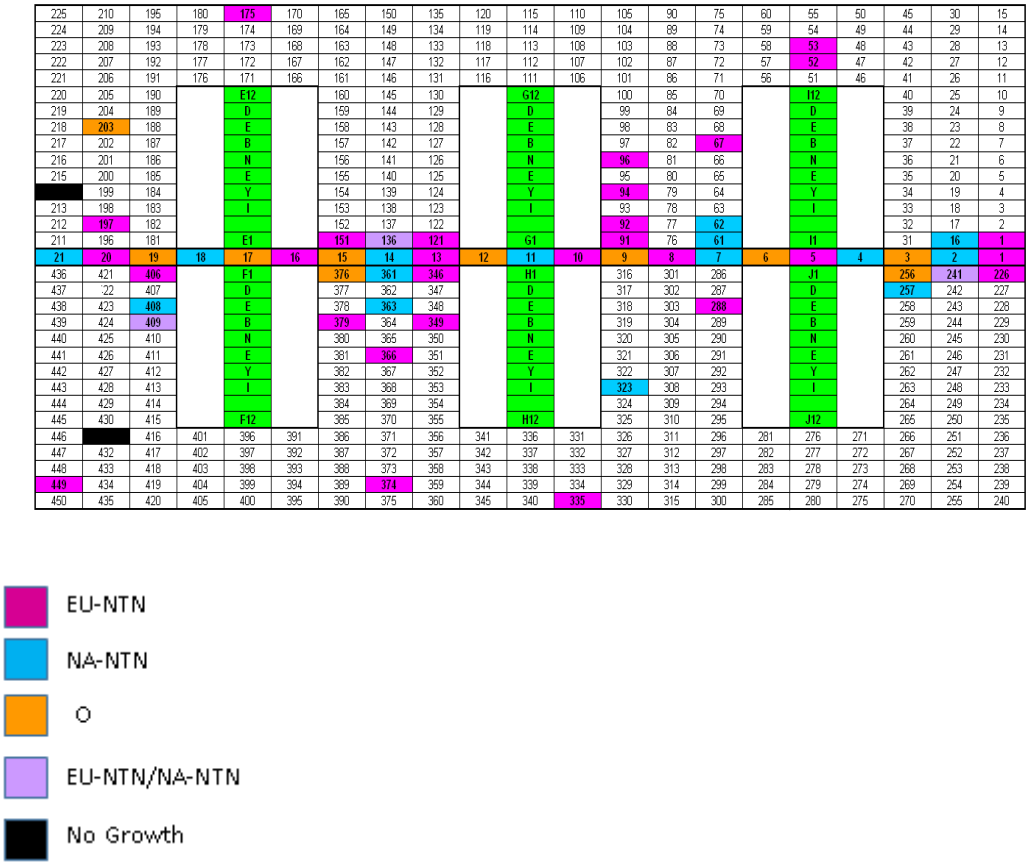


Figure 4.6 Spatial distribution of infected bait potato plants 2010

Different strain types are represented by different colours and the location of each plant is mapped with its position in the field plot. Pink represents PVY^{EU-NTN}, blue is PVY^{NA-NTN}, and orange is PVY^{O}. A lilac colour indicates that the plant is infected with both PVY^{EU-NTN} and PVY^{NA-NTN}.

In total 35 of the 450 bait potato plants were infected with PVY. Of these 21 of the 35 infected plants were found within a 3 plant distance from the infector plants (up to 0.75m) suggesting that the transmission range of the PVY from

the infector plant is relatively short. The plants infected towards the edge of the plot are mostly $PVY^{EU\text{-}NTN}$ which could suggest that the EU-NTN strain travels farther than other strains though this would need extra investigation to prove.

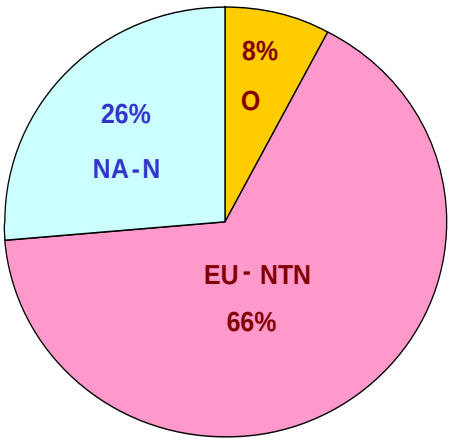


Figure 4.7: Relative proportion of PVY variants found in infected bait potato plants at post-harvest (2010).

Virus incidence on potato bait plants was assessed by testing a bulk of tubers for each plant. PVY infected plants were tested by RT-PCR using primers specific for $PVY^{EU\text{-}NTN}$ and $PVY^{NA\text{-}NTN}$ isolates (Figure 4.7). A significantly higher proportion of plants (66%) were found to be infected by the $PVY^{EU\text{-}NTN}$ isolate, while only 26% and 8% were infected by $PVY^{NA\text{-}NTN}$ and PVY^{O} respectively (Figure 4.8). These results suggest that despite a comparable weekly timing of transmission for the three PVY strains studied, $PVY^{EU\text{-}NTN}$ has the highest propensity to spread and/or to translocate to daughter tubers.

4.3.2 Frequency and timing of transmission of PVY isolates in 2011

4.3.2.1. Weekly frequency of transmission

In 2011 transmission of PVY was observed from week 2 (week beginning 8th June), at this point aphid vector pressure measured only 2. This transmission was 2 weeks earlier than in 2010. The peak in transmission for both PVY^O and PVY^N occurred at week 6 (week beginning 6th of July) where 76% of the 148 *N. debneyi* plants were infected with PVY^N and 41% were infected with PVY^O. Aphid vector pressure peaked at week 8 (beginning 20th of July) with a value of 338, concomitantly in this week a decrease in transmission was observed. Throughout the season the transmission of both PVY^N and PVY^O followed a similar pattern. Overall 74% of infected *Nicotiana debneyi* plants tested positive for PVY^N while 26% had PVY^O where an expected distribution would be 67% to 33%. In the last 4 weeks of the 2011 trial the distribution was in accordance with the expected rate. Early season transmission is considered to have greater impact than late season transmission (Kirchner *et al*, 2011) for several reasons early transmission allows greater opportunity for the virus to spread throughout the primary infected plant to infect progeny tubers, also as plants age they develop a mature plant resistance, where they are less susceptible to infection by viruses. Mature plant resistance to PVY can occur as early as four to six weeks post emergence (Gray and Lampert, 1988).

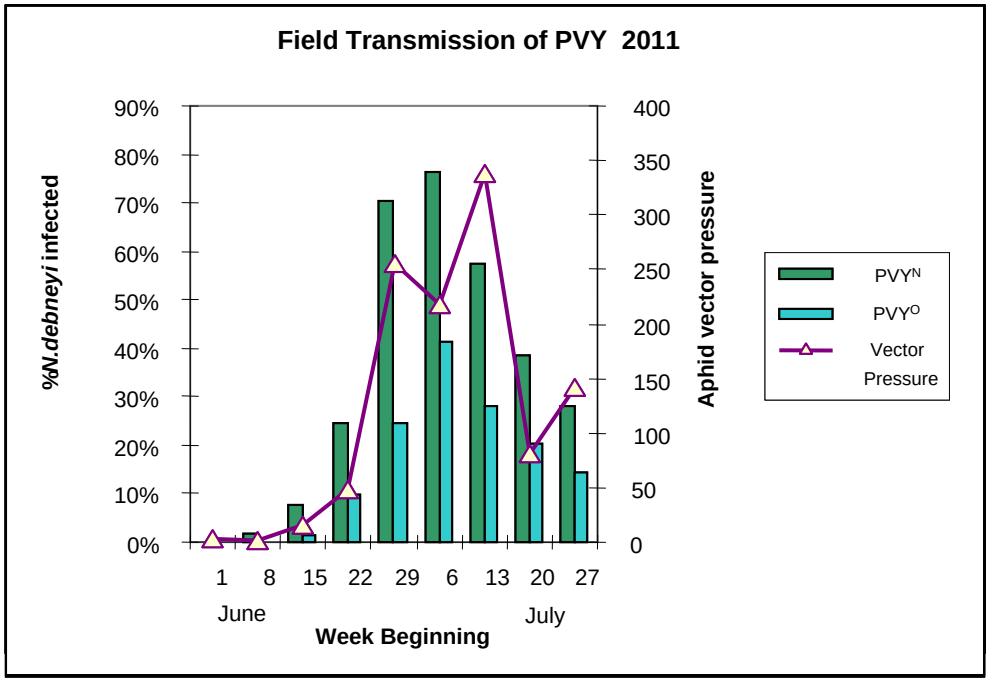


Figure 4.8 Weekly frequency of transmission of PVY^{O} and PVY^{N} serotypes (2011 season). The frequency of transmission is expressed as the percentage of ELISA-positive *Nicotiana debneyi* plants per week. The weekly PVY aphid vector pressure (Gogarbank suction trap, Edinburgh, UK) is indicated.

4.3.2.2. Timing and frequency of transmission of PVY$^{EU\text{-}NTN}$ and PVY$^{NA\text{-}NTN}$ isolates

For all *Nicotiana debneyi* plants which tested positive for PVY^{N} in 2011, RT-PCR was carried out to investigate which variant of PVY^{N} was present, either PVY$^{NA\text{-}NTN}$ (DV69) or PVY$^{EU\text{-}NTN}$ (DV76). Figure 4.9 shows the number of plants infected with each variant. In any plant that tested positive for both

variants of the virus this would be counted twice, once for each variant. Overall there is a clear majority of transmission of EU-NTN, which is statistically significant to P< 0.000001 using the M^{C}Nemar's test for related samples.

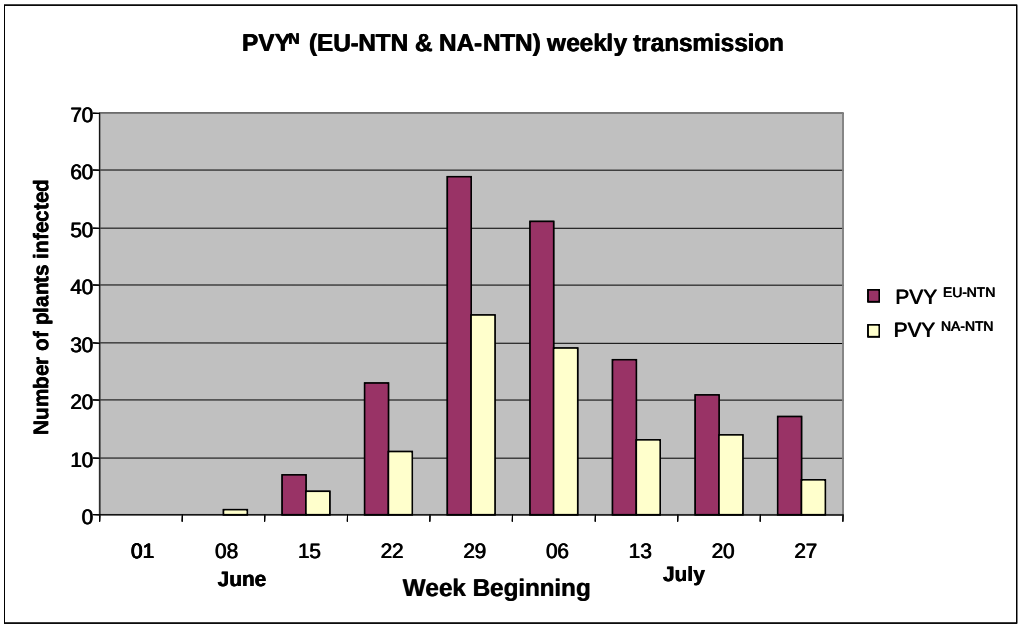


Figure 4.9: Number of PVY^{N} ELISA-positive *Nicotiana debneyi* cases infected with PVY^{EU-NTN} and/or PVY^{NA-NTN} 2011. Mixed infection cases with PVY^{O} were not included in this study.

Weekly transmission monitoring of PVY^{EU-NTN} and PVY^{NA-NTN} was undertaken by molecular genotyping of the PVY^{N} ELISA-positive tobacco plants as described in section 4.2.5. Results presented in Figure 4.9 indicate that weekly timing of transmission of PVY^{EU-NTN} is comparable to PVY^{NA-NTN}, starting at week 3 (week beginning 15th June) (low levels of PVY^{NA-NTN} were

detected at week 2), reaching a maximum at week 5 (week beginning 29 June) and decreasing further on until week 9 (week beginning 27th July) of the trial. A higher number of cases of $PVY^{EU\text{-}NTN}$ were found all through the duration of the experiments with a maximum of 59 and 33 cases at week 5 for $PVY^{EU\text{-}NTN}$ and $PVY^{NA\text{-}NTN}$ respectively. Overall 66% of cases were $PVY^{EU\text{-}NTN}$ and 34% $PVY^{NA\text{-}NTN}$. This suggest that $PVY^{EU\text{-}NTN}$ is transmitted to a higher frequency (approximately 2-fold) than $PVY^{NA\text{-}NTN}$ and suggest that similar aphid species are likely to be responsible for transmitting the different isolates.

4.3.2.3 Distribution and incidence of PVY isolates in field conditions in 2011

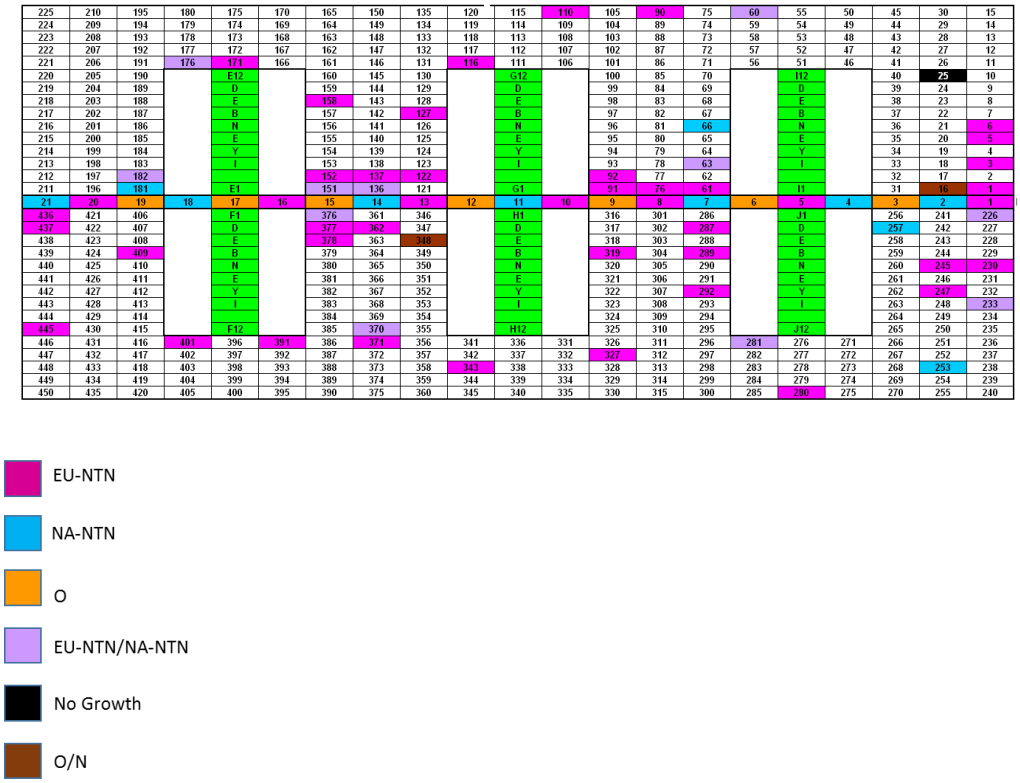


Figure 4.10: Location in the field plot of infected bait potato plants 2011.

Different strain types are represented by different colours and the location of each plant is mapped with its position in the field plot as previously described (pink: $PVY^{EU\text{-}NTN}$, blue: $PVY^{NA\text{-}NTN}$, orange: PVY^{O}). Dual infections are shown as a combination of two colours, lilac represents infection of $PVY^{EU\text{-}NTN}$ and $PVY^{NA\text{-}NTN}$ while brown indicates a dual infection of PVY^{N} and PVY^{O}. In 2011, 54 plants of 450 were found to be infected with PVY (overall incidence of 12%).

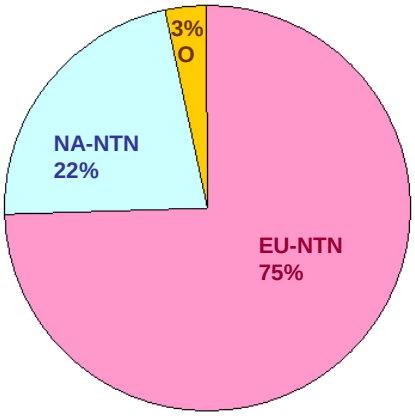


Figure 4.11: Relative proportion of PVY variants found in infected bait potato plants at post-harvest (2011).

Overall in 2011 there was approximately 2-fold more PVY transmission than in 2010, despite vector pressure levels being approximately 2-fold lower than 2010. This year, $PVY^{EU\text{-}NTN}$ was transmitted at a higher rate than for the other two PVY isolates in *Nicotiana debneyi* plants. Incidence of PVY in daughter tubers indicates that 75% of infected tubers were infected with $PVY^{EU\text{-}NTN}$.

4.3.3. Frequency and timing of transmission of PVY isolates in 2012

4.3.3.1. Weekly transmission to tobacco bait plants

In 2012 transmission of PVY was observed from week 3 (week beginning 20th June), at this point aphid vector pressure measured 120. PVY transmission started 2 weeks later than in 2010. The peak in transmission for PVY^{N}

occurred at week 4 (week beginning 27th of June) and week 9 (week beginning 1st of August) where 16% of the 148 *N. debneyi* plants were infected with PVY^N. The highest transmission rate for PVY^O was observed in week 5 (week beginning 4th July) where 5 % of all *N. debneyi* plants were infected. Aphid vector pressure peaked at weeks 3, 5 and 9 with values of 120, 213 and 147 respectively. The peaks in aphid vector pressure corresponded with higher rates of infection of tobacco plants. Throughout the season the transmission of both PVY^N and PVY^O followed a similar pattern with the exception of week 4 where PVY^O doubled, while for PVY^N there was a 10-fold increase in transmission observed. This indicates that for this week only one species of aphid present may have been able to transmit only PVY^N or to transmit it far more effectively than PVY^O. Overall 80% of infected *Nicotiana debneyi* plants tested positive for PVY^N while 20% had PVY^O where an expected distribution would be 67% to 33%. The greatest difference in transmission was at week 4 where PVY^N accounted for 90% of transmission.

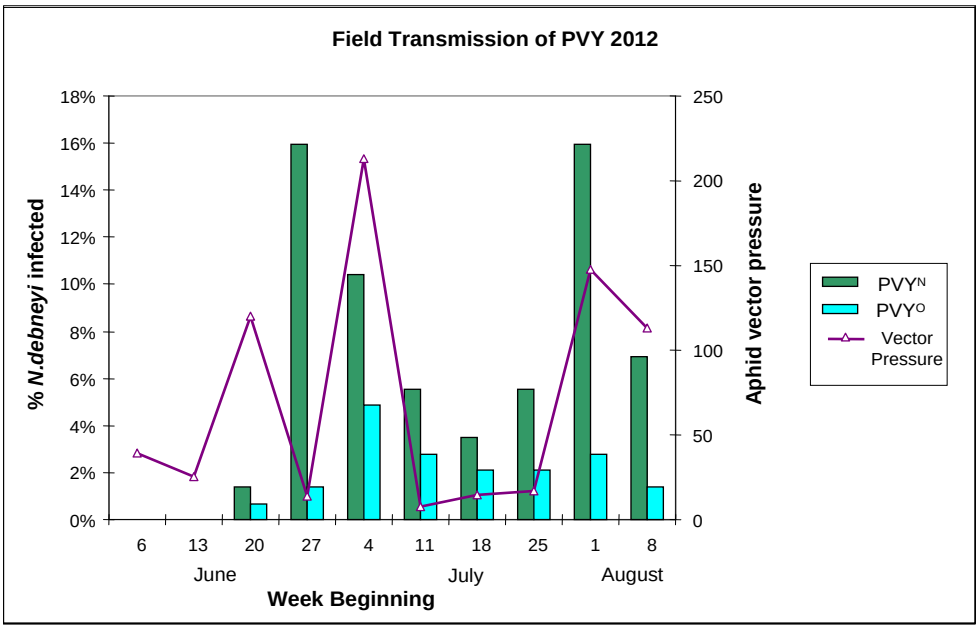


Figure 4.12: Weekly frequency of transmission of PVY^{O} and PVY^{N} serotypes (2012 season). The frequency of transmission is expressed as the percentage of ELISA-positive *Nicotiana debneyi* plants per week. The weekly PVY aphid vector pressure (Gogarbank suction trap, Edinburgh, UK) is indicated.

4.3.3.2. Timing of transmission of PVY$^{EU\text{-}NTN}$ and PVY$^{NA\text{-}NTN}$ isolates

Weekly transmission monitoring of PVY$^{EU\text{-}NTN}$ and PVY$^{NA\text{-}NTN}$ was undertaken by molecular genotyping of the PVY^{N} ELISA-positive tobacco plants as described in section 4.2.4). Results presented in Figure 4.13 indicate that weekly timing of transmission of PVY$^{EU\text{-}NTN}$ is comparable to PVY$^{NA\text{-}NTN}$, starting at week 3 (week beginning 20th June) (low levels of PVY$^{NA\text{-}NTN}$ were detected at week 5), peaks of transmission were at week 4 and 9, however the

peak at week 9 was almost entirely from PVY^{EU_NTN}. A higher number of cases of PVY$^{EU\text{-}NTN}$ were found all through the duration of the experiments with a maximum of 15 and 8 cases at week 5 for PVY$^{EU\text{-}NTN}$ and PVY$^{NA\text{-}NTN}$ respectively. Overall 75% of cases were PVY$^{EU\text{-}NTN}$ and 25% PVY$^{NA\text{-}NTN}$. This suggests that a PVY$^{EU\text{-}NTN}$ is transmitted to an even higher frequency in 2012 (3-fold) than PVY$^{NA\text{-}NTN}$.

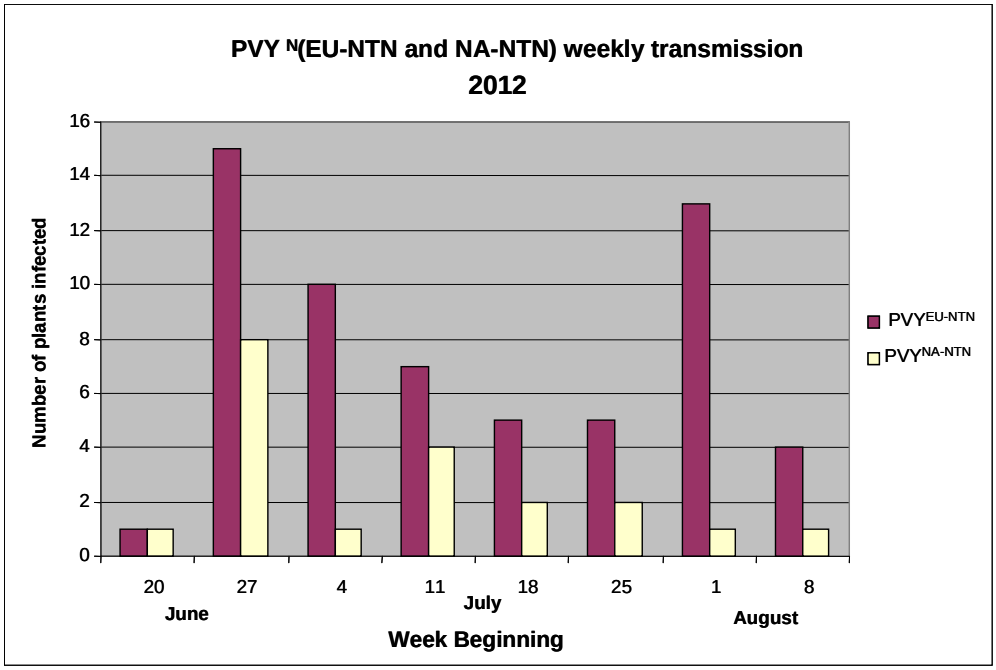


Figure 4.13 Number of PVY^{N} ELISA-positive *Nicotiana debneyi* cases infected with PVY$^{EU\text{-}NTN}$ and/or PVY$^{NA\text{-}NTN}$ 2012.

4.3.3.3. Incidence and spatial distribution of PVY infection in potato bait plants

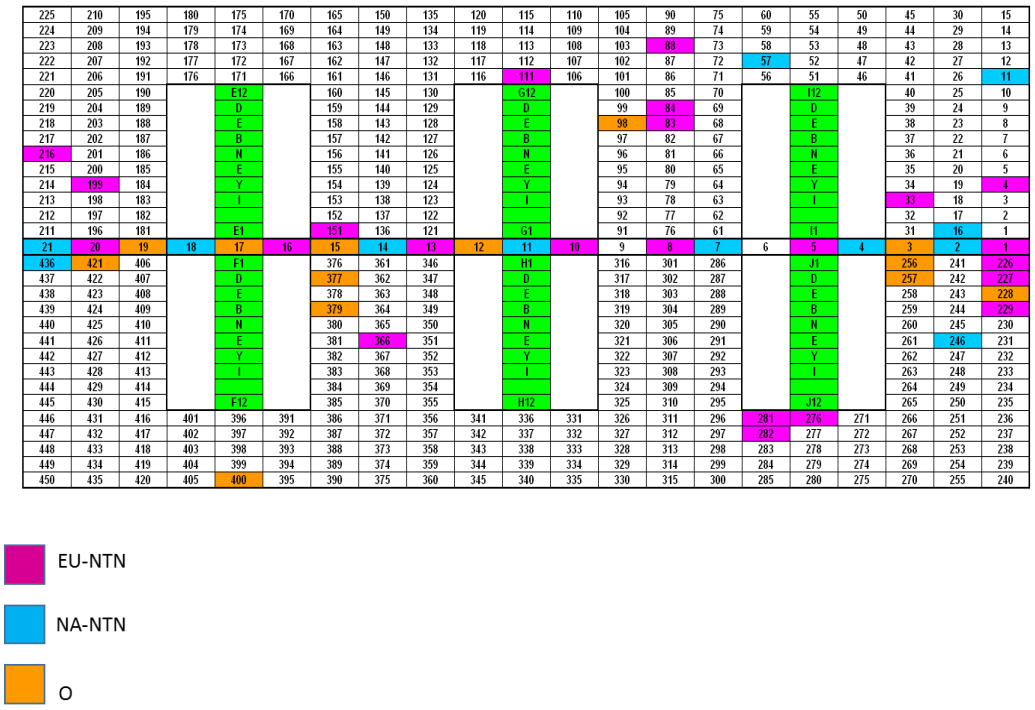


Figure 4.14: Location in the field plot of infected bait potato plants 2012

Different strain types are represented by different colours and the location of each plant is mapped with its position in the field plot as previously described (pink: $PVY^{EU\text{-}NTN}$, blue: $PVY^{NA\text{-}NTN}$, orange: PVY^{O}). No dual infection occurred in 2012.

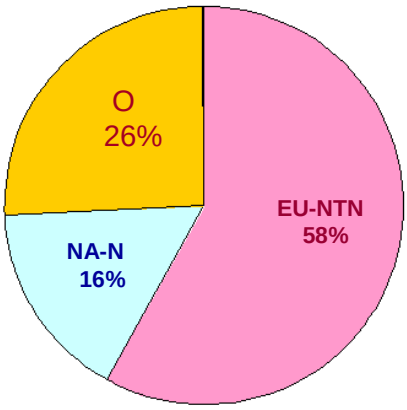


Figure 4.15: Relative proportion of PVY variants found in infected bait potato plants at post-harvest (2012). In 2012, 29 plants out of 450 were infected by PVY (6.5% PVY incidence). $PVY^{EU\text{-}NTN}$, PVY^{O} and $PVY^{NA\text{-}NTN}$ represented 58%, 26% and 16% of cases respectively.

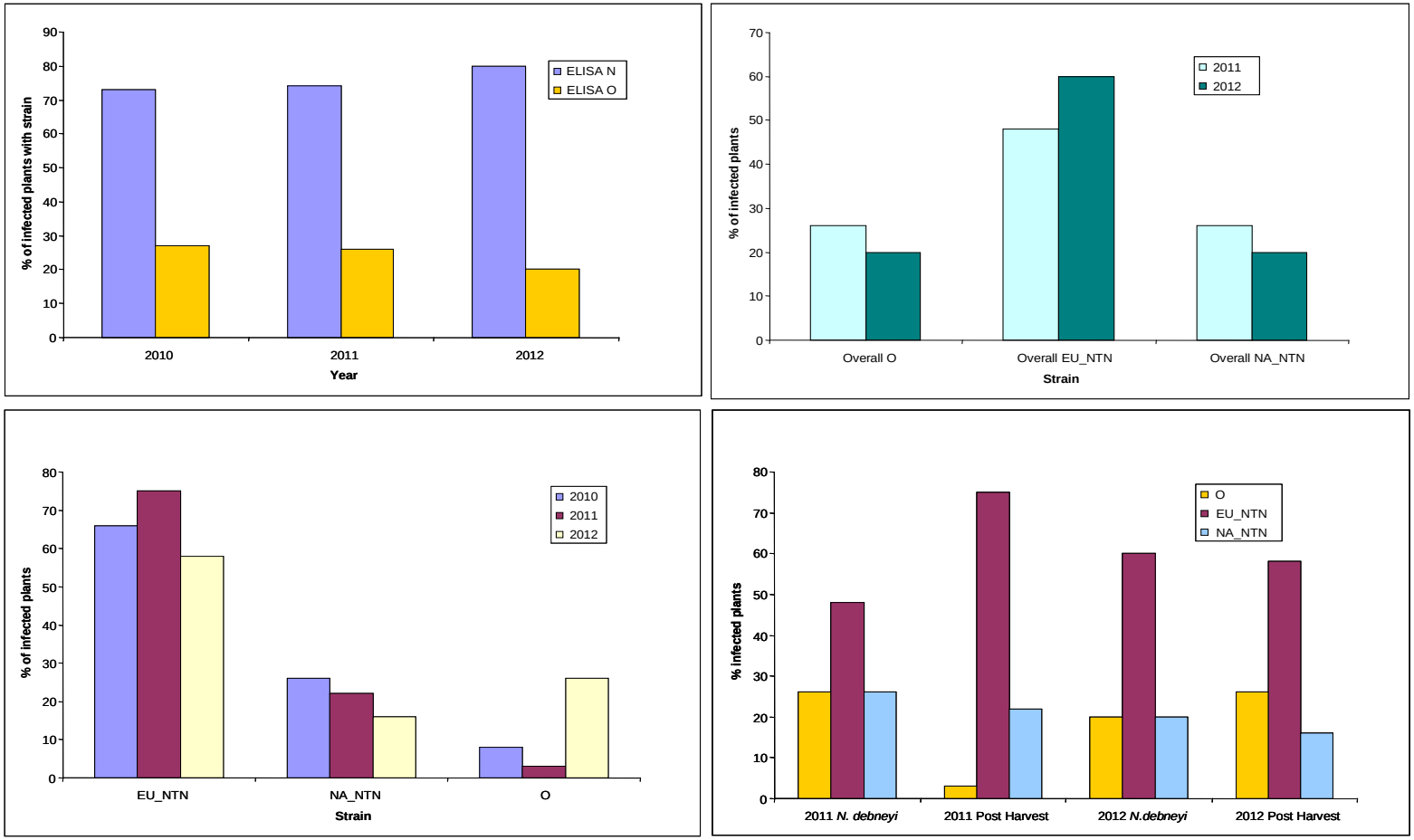


Figure 4. 16: Summary bar charts for the three trial years. Top left: weekly infection of *Nicotiana debneyi* plants by PVY^{O} and PVY^{N} serotypes. Bottom left: Yearly PVY incidence in tubers for each isolate. Top right: Percentage infection for each isolate in *N. debneyi* for 2011 and 2012. Bottom right: Relative incidence for each isolate in *N.debneyi* and potato at post-harvest (2011 and 2012).

4.3.4 Summary of field trial

Overall, data obtained from three years of field trials suggest that PVY^{N} serotypes are transmitted at a higher frequency than PVY^{O} (~75% to 25% respectively). The expected transmission rates would be 67% PVY^{N} to 33% PVY^{O}. For 2011 and 2012 molecular typing of the isolates revealed a dominance of PVY^{EU_NTN}, which is found in ~2-fold more *N. debneyi* bait plants compared to $PVY^{NA\text{-}NTN}$ isolate. In 2010 and 2011 the amount of potato plants infected by $PVY^{EU\text{-}NTN}$ was greater than might be anticipated according to weekly transmission experiments, thus implying that aphid-transmission frequency of PVY isolates might not be the main factor responsible for PVY incidence levels in tubers. End of season distribution in harvested tubers was up to 32 times the amount of PVY^{N} serotype to PVY^{O} serotype. In all three years $PVY^{EU\text{-}NTN}$ was the largely prevalent isolate accounting for at least 58% of infected plants; while the expected incidence would be of 33%.

4.3.5 Analysis of interdependence in transmission of PVY strains

In order to assess the distribution of viruses over their potato hosts in the field and determine if there are potential associations between specific virus isolates within the same plant. The patterns of infections by the three different PVY strains were studied. The potential relationship between transmission of PVY^{O}, $PVY^{EU\text{-}NTN}$ and $PVY^{NA\text{-}NTN}$ was assessed (*Chi*-square analysis method, Fowler *et al*., 1998). The results reported in Table 4.3 indicate that when *Chi*-square analysis was carried out to test for independence between PVY^{O} and PVY^{N} (at the serological level) a highly significant interaction that there is a strong relationship between PVY^{O} and PVY^{N} transmission in 2010 and 2011

but not 2012. The actual number of mixed infections was higher (27,159) than the expected number (7, 74) in both 2010 and 2011 respectively. This was particularly notable with PVY^{O} where in 2011 there were nearly four times as many dual infected PVY^{O}-PVY^{N} (159) *N. debney*i plants than single PVY^{O} (45) infected plants. Likewise, assessing in a similar way interactions between $PVY^{EU\text{-}NTN}$ and $PVY^{NA\text{-}NTN}$ strains, a highly significant relationship was again found between these isolates (belonging to the N-serotype). Again this was particularly notable for $PVY^{NA\text{-}NTN}$ where twice more plants were dual infected $PVY^{NA\text{-}NTN}$ and $PVY^{EU\text{-}NTN}$ (76) than individually $PVY^{NA\text{-}NTN}$ (39) infected plants. Contrastingly, for $PVY^{EU\text{-}NTN}$, approximately half the number of plants were dual infected ($PVY^{EU\text{-}NTN}$ and $PVY^{NA\text{-}NTN}$) than the numbers of singly $PVY^{EU\text{-}NTN}$ (131) infected plants. Overall an interaction was observed between the transmission of PVY^{O}, PVY^{N} and $PVY^{NA\text{-}NTN}$. $PVY^{EU\text{-}NTN}$ was generally found within each of the PVY^{O} and $PVY^{NA\text{-}NTN}$ cases suggesting a strong association of $PVY^{EU\text{-}NTN}$ with any of the other PVY strains. Multiple infections lead to a variety of intrahost virus-virus interactions (Syller, 2012). Overall, this skewed distribution towards PVY^{N} and in particular $PVY^{EU\text{-}NTN}$ suggest that during multiple infections, PVY^{O} and $PVY^{NA\text{-}NTN}$ might facilitate $PVY^{EU\text{-}NTN}$ super-infection and $PVY^{EU\text{-}NTN}$ infection might antagonise further PVY^{O} and $PVY^{NA\text{-}NTN}$ super- infection of the same host plant

Table 4.3: Expected and actual distribution of mixed infections including *Chi*-square analysis and *P*-values to show strength of relationships.

	Expected distribution	Actual distribution	Chi squared value	P.value
PVY^{O}/ PVY^{N} dual infection 2010	7	27	59.98	<0.001
PVY^{O}/ PVY^{N} dual infection 2011	74	159	179.88	<0.001
$PVY^{NA\text{-}NTN}$/ $PVY^{EU\text{-}NTN}$ dual infection 2011	18	76	226.65	<0.001
PVY^{O}/ PVY^{N} dual infection 2012	2	0	3.00	>0.05
$PVY^{NA\text{-}NTN}$/ $PVY^{EU\text{-}NTN}$ dual infection 2012	1	15	220.81	<0.001

4.3.6 Analysis of interaction between different PVY isolates: *in planta* translocation.

An assessment of the relative fitness of $PVY^{NA\text{-}NTN}$, PVY^{O} and $PVY^{EU\text{-}NTN}$ isolates in single and mixed infections was undertaken, in order to understand whether or not within plant competition occurs between different isolates and if so, which isolate(s) are most successful in infecting progeny tubers in these conditions.

Table 4.4 Results from dual inoculation of plants with different isolates

	Post Harvest Tuber Test % present each isolate				
Inoculated with	DV76	DV69	DV71	Negative	Overall Dominance
76, 69	100	50			76
76, 71	50		100		71
69, 71		100	33		69
2x76,69	33			67	76
2x69,76	75	50			76
2x76,71	75			25	76
2x71,76	75		50	25	76
2x69,71		25	50	25	71
2x71,69		75	100		71
10x76,69	50	50			none
10x69,76	75	50			76
10x76,71	75		25		76
10x71,76			100		71
10x69,71			50	50	71
10x71,69			100		71
Aphid 76,69				100	0
Aphid 76,71				100	0
Aphid 69,71				100	0
76 Control	100				76
69 Control		100			69
71 Control			100		71
Negative Control				100	0

The PVY isolates used and their relative inoculum titre (1x-2x-10x) is indicated in the left, where 2x this means that the isolate at 2x is concentrated and the other isolate is a 1:1 dilution, where 10x, the 10x isolate is concentrate

and the other isolate is 1:9 dilution. The isolates used were DV69=$PVY^{NA\text{-}NTN}$, DV71=PVY^{O} and DV76=$PVY^{EU\text{-}NTN}$. The percentage of plants infected by each isolate is indicated in the column under the isolate identity. 100% means all plants are infected with this specific isolate. Where a plant failed to emerge this was not included.

Maris Piper plants were inoculated with different concentrations of PVY isolates as indicated in (Table 4.4). Four biological replicates per inoculum combination were used and growing-on DAS-ELISA test was performed on the progeny tubers as previously described. Where all four plants tested positive for a particular isolate is was given a score of 100%, the isolate that infected the highest percentage of plants in each inoculation combination was counted as "dominant" isolate (reported as "Overall Dominance". In these controlled conditions, DV76 ($PVY^{EU\text{-}NTN}$) was dominant on 7 occasions and DV71 (PVY^{O}) was dominant on 6 occasions. This preliminary investigation would need to be further developed to draw statistically significant conclusions. However, with this limited dataset, early observations suggest that when multiple infection occur, both PVY^{O} and $PVY^{EU\text{-}NTN}$ appears to outcompete $PVY^{NA\text{-}NTN}$. No apparent threshold of inoculum titre was a driver of this prevalence, as even with a ten-fold titre difference, the isolate with the lower inoculum titre can still out-compete the isolate with the highest inoculum titre based on incidence in progeny tubers. The experiment was also attempted through aphid inoculation where *Myzus persicae* fed on infected *Nicotiana tabacum* plants prior to feeding on healthy Maris Piper plants, none of these inoculations were successful on this occasion.

4.4 Discussion

4.4.1 Aphid transmission

Over the course of the 3 year trial, PVY serotype N was found to be transmitted more effectively than expected. This serotype was detected in 70 to 80% of infected *N. debneyi* plants across a growing season. The expected distribution would be 67% PVY^{N} to 33% PVY^{O}.

For 2011 and 2012 molecular typing of the isolates revealed a dominance of $PVY^{EU\text{-}NTN}$, which is found in at least twice as many *N. debneyi* plants as $PVY^{NA\text{-}NTN}$ strain. These results show that the $PVY^{EU\text{-}NTN}$ strain is the most effectively transmitted strain by aphids across the trial period. Nucleotide changes in the HC-Pro and the coat protein region of the virus genome could lead to greater acquisition and deposition of the virus, which could explain increased levels of transmission. In our trials, aphid species responsible for the transmission might have been similar but $PVY^{EU\text{-}NTN}$, (isolate DV76) due to its genetic structure, is more readily aphid-transmitted than other PVY species. There is also the possibility that virus infection can induce subtle chemical modifications of the infected host plant which could consequently influence aphid behaviour. It has not yet been investigated if different strains of the virus affect aphid behaviour differently however alterations of host plant chemistry by the virus could provide an explanation in to why one strain of the virus is more readily acquired and therefore transmitted by the aphid vector. Mauck *et al* (2010) demonstrated that cucumber mosaic virus (CMV) reduced the host plant quality causing aphids to disperse quickly after feeding on the plant, along with this however the virus caused the plants to emit volatiles similar to

that of a healthy plant in order to attract the aphid, these two factors optimised the spread of a non-persistent virus. If PVY is having a similar effect on plants it is possible that the plant volatiles are affected differently by different strains. Boquel *et al.* (2011) found that PVY did affect *Myzus persicae* behaviour in relation by increasing feeding activity and colonisation. This change in behaviour is beneficial to the virus as this is a sedentary species with a high vector efficiency to transmit PVY. When aphids numbers on the plant reach critical levels, winged offspring are produced that can in turn transmit PVY. Contrastingly for *Macrosiphum euphorbiae,* which is a more motile aphid and less effective virus transmitter, feeding activity was reduced in PVY infected plants. In this case, the aphid was more likely to leave the plant, which again would be beneficial for the virus as by this method it is likely to be rapidly transmitted.

Another possibility is that different aphid vectors are responsible for the transmission of different strains. Verbeek *et al*. (2010) found differences in transmission efficiencies of isolates under controlled conditions. Different aphid species peak in numbers at different times according to their lifecycle and migration to new host plants so in order to get an indication of whether different aphid species are responsible for virus transmission in the field the timing and peaks of transmission of strains could be assessed. Overall for the three year study discussed in this chapter the timing and peaks of transmission occur at a similar point between the three PVY strains used, suggesting that similar aphid species are responsible for their transmission. There is however one notable exception between $PVY^{EU\text{-}NTN}$ and $PVY^{NA\text{-}NTN}$ in 2012 which was

a particularly low aphid vector year were particularly low levels of *M. dirhodum* and *S. avenae* were caught, but higher than average levels of aphids thought to be less important such as *C. aegopodii*. In 2012 year peaks in $PVY^{EU\text{-}NTN}$ occur where peaks in $PVY^{NA\text{-}NTN}$ do not. This would imply that in the absence of the main PVY vector species (in Scotland *M. dirhodum*, *S. avenae* and possibly *R. padi,* Pickup *et al*., 2009) there may be differences in the aphid species responsible for the transmission of different strains. One anomaly that occurred was that the aphid vector pressure was highest in 2010 however the highest rate of transmission was in 2011. The composition of aphids was different between these years and this might suggest that the vector indices may not be entirely accurate when environmental factors are taken in to account. Alternatively it may have been a local effect in the vicinity of the plot itself. Low virus levels were observed in the growing crop inspections in the subsequent year (2012), suggesting that the low aphid numbers in 2011 had reduced transmission generally, despite this contradicting what was observed in the field trial.

4.4.2 Incidence and distribution of PVY variants in the potato crop

In all years, the number of potato plants infected by $PVY^{EU\text{-}NTN}$ was greater than expected. Virus incidence and distribution in harvested tubers was up to 32 times the amount of PVY^{N} serotype to PVY^{O} serotype. In all three years the prevalent strain was $PVY^{EU\text{-}NTN}$ accounting for at least 58% of infected plants. This observed $PVY^{EU\text{-}NTN}$ incidence is significantly different from the expected distribution of 33% for a similar number of infector plants for each isolate. The field trail discussed in this chapter used the cultivar Maris Piper as

it is historically the predominant crop grown in Scotland and it also susceptible to both PVY^{N} and PVY^{O}. The only variation in the use of cultivar was in 2010 where $PVY^{EU\text{-}NTN}$ was only available from infected Nadine plants. These Nadine infectors displayed very strong necrotic symptoms by 6 to 7 weeks in the field resulting in the complete necrosis of the plant and ultimately loss of three $PVY^{EU\text{-}NTN}$ infectors by 8 weeks after planting. It was therefore surprising to observe a higher frequency of $PVY^{EU\text{-}NTN}$ transmission in spite of the detrimental effect of $PVY^{EU\text{-}NTN}$ infection in these plants and ultimately the loss of some of these Nadine infectors. This suggests that, even with a reduced number of $PVY^{EU\text{-}NTN}$ infectors (down to 4 infectors by week 6 instead of 7 as for the other PVY), transmission levels of $PVY^{EU\text{-}NTN}$ might have been underestimated in 2010. The replacement of cultivar Nadine as the source of inoculum for $PVY^{EU\text{-}NTN}$ in 2010 to Maris Piper did not result in obvious differences in the relative transmission frequency of this variant suggesting that cultivar choice had a negligible effect.

In order to prevent generational changes in the molecular nature of the virus (e.g. potential loss of aphid transmissibility) tubers used for inoculum were selected from Maris Piper plants infected the previous year with the virus. However in the case of PVY^{O} two of the tubers planted tested negative for virus despite other tubers from the same plants testing positive the year before. Therefore, in the final year, PVY^{O} transmission increased despite the lower inoculum levels. The reasons for this relative increase in PVY^{O} incidence are unknown. In 2012 compared to previous years, a much lower aphid vector pressure was observed than the previous years for which different populations of aphid species were accounting for the overall PVY vector pressure.

Contrastingly, because in 2010 and 2011 comparable frequency of transmission and virus incidence was observed both in potato bait plants and *N. debneyi* might imply that in years of high aphid vector pressure, other factors are involved in the spread of the PVY to the tuber.

Differences in the transmission rate between the different isolate strains may not account for the overall prevalence of $PVY^{EU\text{-}NTN}$ in tuber progeny. A more likely hypothesis is that $PVY^{EU\text{-}NTN}$ is more effective at replication and systemic infection throughout the plant resulting in an optimum translocation to tubers. Weidemann (1988) stated the results of several studies on translocation of PVY^{N} within potato plants have shown that the translocation of PVY^{N} is much faster than that of PVY^{O}. Consequently, if $PVY^{EU\text{-}NTN}$ is the most efficient variant with regards to intraplant movement, it may become more readily available for aphid transmission. This could result in a higher source of $PVY^{EU\text{-}NTN}$ inoculum within the growing season. This could partially explain the prevalence of $PVY^{EU\text{-}NTN}$ in tubers and its overall prevalence in seed potatoes. Overall the results from the field trial suggest that $PVY^{EU\text{-}NTN}$ is the fittest strain under field conditions.

4.4.3 Dual infection of bait plants

In order to assess the significance of the difference in transmission of the different strains it was first important to assess potential association between PVY species and whether all three PVY species behaved independently from each other. The relative frequency of transmission for each PVY species was analysed using Chi Squared method. The results showed a strong association

(*i.e.* occurrence of mixed infections) between PVY isolates during aphid-transmission and that this relationship varied from year to year. In 2010 and 2011 there was a strong association between PVY^{N} serotype and PVY^{O} serotype, molecular testing was not included in this year and it was therefore not possible to assess $PVY^{EU\text{-}NTN}$ and $PVY^{NA\text{-}NTN}$ counts and level of mixed infection. In 2011 there was a strong association between PVY^{O} and PVY^{N} serotypes and between $PVY^{EU\text{-}NTN}$ and $PVY^{NA\text{-}NTN}$ genotypes. It was not possible to test the association between the PVY^{O} and PVY^{N} genotypes in this study due to a cross amplification of the $PVY^{EU\text{-}NTN}$ primers with PVY^{O}. In 2012 there was no relationship between PVY^{O} and PVY^{N} but a strong relationship was observed between $PVY^{EU\text{-}NTN}$ and $PVY^{NA\text{-}NTN}$. Post-harvest testing of infectors' tuber progeny revealed a high proportion of mixed infection (up to 90% in some cases). Interestingly, plants that were dual infected usually included PVY^{EU_NTN} with either $PVY^{NA\text{-}NTN}$ or PVY^{O}. Possible explanations for the high proportion of dual infection might be due to the close proximity of each infector plants thereby increasing the possibility for incoming aphids to acquire more than one PVY isolate before moving to the next bait plant and may therefore transmit more than one strain simultaneously. The other possibility is that, as mentioned previously, the infected plant releases volatiles attracting aphids, thereby increasing the risk of dual infection. It is notable that where a dual infection occurs, the vast majority of PVY cases include $PVY^{EU\text{-}NTN}$ with any other isolate combination. Such trend was not observed in independent trials where infectors with single PVY isolate/strain are separated into different plots, (unpublished work SASA). The fact that $PVY^{EU\text{-}NTN}$ is the predominant isolate in infected potato

bait plants might suggest that $PVY^{EU\text{-}NTN}$ out competes other isolates in dual infection. This apparent fitness represents a selective advantage for infecting progeny tubers therefore, making it more likely to present a source for secondary infection.

4.4.4 Intra-plant competition

As stated, there is a highly significant interaction between different PVY variants in our field trials, which leads to a high proportion of mixed infections. There is a discrepancy between the number of virus cases from weekly transmission to *N. debneyi* and virus incidence in potato bait plants at post-harvest, which suggests that the over-representation of $PVY^{EU\text{-}NTN}$ in tubers might be the result of competitions between PVY species. This hypothesis implies that when there is a mixed infection in the plant the $PVY^{EU\text{-}NTN}$ variant can out-compete other variants and will more readily translocate to daughter tubers. Amaku *et al* (2010) modelled competition between two different Barley yellow dwarf virus (BYDV) strains where one strain had replaced the other. They found that although there were differences in aphid transmission, the most important factor for the displacement of one strain by another was competition within the plant where one strain prevailed over the other strain. To test this hypothesis, preliminary experiments were conducted using both manual inoculation of two PVY strains and aphid inoculation. Under these conditions aphid transmission of PVY was very low and no conclusions could be drawn from this experimental set-up. The experiments involving manual inoculation of $PVY^{EU\text{-}NTN}$, $PVY^{NA\text{-}NTN}$ and PVY^{O} yielded significant infection levels. While $PVY^{EU\text{-}NTN}$ was readily more detected in

tubers than $PVY^{NA\text{-}NTN}$ no obvious discrepancy between incidence of $PVY^{EU\text{-}NTN}$ and PVY^{O} isolates was observed. This preliminary experiment suggests that within plant competition of the PVY isolates selected might be only observed in the field or where a mixed infection occurs with $PVY^{EU\text{-}NTN}$ and $PVY^{NA\text{-}NTN}$. Environmental conditions may be an important factor for PVY epidemiology and intraplant competition dynamics and therefore it may not be faithfully reproduce in controlled conditions.

4.4.5. Other factors affecting PVY epidemiology

Several parameters will affect PVY epidemiology which is a fine interaction between the host plant, the virus, the vector and the environment. The genetic background of the host plant potato cultivar will have different susceptibilities to PVY with many more cultivars being infected with PVY^{N} than PVY^{O}. The availability of more cultivars which can be infected by PVY^{N} will in itself lead to an increased source of inoculum in the field. Potato plants are known to become more resistant to infection by PVY depending on when they are exposed to infection. Older plants are less likely to become infected and this phenomenon is known as mature plant resistance (Sigvald, 1985, Gibson 1991). Previous studies have shown that in controlled conditions mature plant resistance is more effective against PVY^{O} than PVY^{N} (Beemster, 1987). It maybe that a control strategy for PVY is to plant as early as possible, thereby when the aphid migrations occur the plants are at a later stage of maturity and less susceptible to infection. Including the knowledge of mature plant resistance as this may also partially explain why PVY^{N} is outcompeting PVY^{O} and further studies should be undertaken to investigate whether this effect

could play a key role in PVY epidemiology. The source of inoculum can be a variable factor in PVY epidemiology, with some plants being more resistant or susceptible to aphids, resistant, tolerant or susceptible to virus or susceptible to virus but perhaps a poor source of inoculum (Hollings, 1955). Other *solanaceous* crops and weeds infectible by PVY are potential reservoirs of the virus and may be a problem in some areas (Cervantes and Alvarez, 2011). Weeds have been noted for their importance as virus sources but also for being breeding sources for aphids (Latorre, 1983). Overall though the main source of inoculum is thought to come from the parent crop and neighbouring potato crops. Field studies of virus spreading have demonstrated that primary infection occurs within a relatively limited range (SASA, unpublished work). Neighbouring seed potato crops of lower grade and ware crops that have undergone several field generations can also be a significant source of virus inoculum. In addition, where potato crops have been grown previously in a field there is the risk of volunteer potatoes, which are plants produced by tubers which have not been harvested from the previous crop. These plants may therefore have been *in situ* for many years, increasing the chances of acquiring virus and therefore becoming a source of inoculum.

4.4.6 Control of PVY

When the industry is considering how to control the spread of PVY the complex epidemiology of the virus must be taken in to consideration. The main source of inoculum is from infected tubers. Roguing (removal) of volunteers and symptomatic plants is an important cultural practice that may reduce virus inoculum levels (Ragsdale *et al*., 2001).

Care should be taken to plant high grade seed crops away from lower grade seed or ware which is likely to contain higher virus levels. Our studies have shown that the highest level of infection occurs within a few plants from the original source of inoculum therefore keeping high grade seed a safe distance away from inoculum will help prevent infection. Where lower grade seed or ware crops are grown in close proximity to high grade seed crops barrier crops have been shown to offer a level of protection (DiFonzo *et al*., 1996). The idea is that the aphid picks up the virus on one crop then deposits it in the barrier crop before landing on the seed crop, barrier crops, should, however not be susceptible to PVY or they may instead become a source of inoculum.

An early example of epidemiological research was Hollings (1955) who found that there was a close correlation between the spread of Potato leaf roll virus (PLRV), PVY and the presence of *Myzus persicae*. He suggested that monitoring aphids might be an effective tool to assess virus risk and with weather having a direct effect on the size of aphid populations, stated that growing of virus-free seed potatoes is more achievable in cool-wet windy countries such as Scotland. Today SASA provides an aphid monitoring programme in conjunction with Rothamstead Research which gives advice to growers on the threat of virus using both aphid incidence and virus levels in the previous season. Identification and control of aphid species may have its limitations due to the complexity of the potato-growing areas. Orlob (1962) identified different forms of the same species of aphid (*Aphis nasturtii*) and found that migratory forms were able to transmit PVY while colonising ones were not. In Finland, it was suggested that controlling *Aphis fabae* on winter

populations could lead to a reduction in virus spread in the following season, thus showing that geographical differences lead to a different dominant vector and that controlling one vector species might result in the contribution of another aphid species in its place. Harrington and Gibson (1989) found a statistical relationship between the number of *M. persicae* and *B. helichrysi* and PVY transmission in England. However, in Scotland *M. dirhodum* and *S. avenae* that are thought to be responsible for transmission (Pickup *et al.*, 2009). Alate *M. dirhodum* and *S. avenae* are known to migrate to wheat from the middle of May (Ankersmit *et al.*, 1981) and numbers peak in late July early August (SASA, suction trap data). Early planting of potato cultivars may avoid peak aphid transmission of the virus (Radcliffe and Ragsdale, 2002) and allow the onset of mature plant resistance to have occurred by the aphid peak (Gibson, 1991).

Mineral oils are used widely in many countries (Bell, 1989) to control PVY. These are thought to reduce the transmission of PVY (Gibson and Rice, 1986). It is believed this is done either by modifying the charge of the stylet thus impeding adsorption of the virus particles or by producing a barrier blocking the uptake of the virus. Some authors have also reported that the use of a straw mulch significantly reduces aphid numbers and as a result PVY infection (Saucke & Doring, 2004).

It is important to consider all the environmental complexity and social-economic factors related to controlling the transmission of PVY. An example which displays the level of environmental complexity that should be considered is a study by Hodge *et al*. (2011) where parasitic wasps were used as a biological control of aphids and it was found that while the wasp caused a reduction in the aphid numbers it also caused a 9 fold increase in virus infection. They hypothesised that disturbance by the parasitoids resulted in more aphid movement and probing and they suggest that the effect of parasitoids on virus dispersal should be considered when setting epidemiological models. An example of socio economic factors would the management of cereal aphids in neighbouring crops if no direct benefits are to be perceived (*i.e.* for example why should cereal farmers control cereal aphids if the only benefit is associated with the protection of neighbouring potato crops). Moving towards more appropriate virus control strategies is only possible if economic interests of stakeholders are in line with changing current practices (Doring 2011).

Potato cultivars display different levels of susceptibility to PVY and therefore the genetic background of the seed potato cultivated will have an impact on the epidemiology of PVY. Several resistance genes that confer extreme resistance or hypersensitive resistance to PVY (PVY^{O} and PVY^{N}) resistance has been identified (Szajko *et al.,* 2008). However, the selection of cultivars are often effectuated on the basis of market demands (processing qualities, taste), agronomical traits (yield), resistance to other pathogen and pests (such as nematodes, bacterial and fungal diseases) and often displaying low to

moderate levels of resistance to PVY. In addition, PVY^{O} is traditionally used as a source of inoculum in the assessment of disease resistance to potato varieties. Disease resistance assessment of potato varieties to PVY^{N} serotype is not performed as standard, which highlights the risk of potential selection of PVY^{N} susceptible cultivars in the seed certification scheme as is now the most prevalent strain of PVY.

4.5. Conclusions

The epidemiology of PYV is a multitrophic interaction between virus-host,-vector-environment and the management of viruses and cultural practices of the grower. Variable factors affecting the crop include differences in varietal susceptibility to virus, the initial incidence of the virus, the geographical environmental conditions, cultural practices within agriculture and also the fitness of the virus taking into account all of the variables.

This study investigated the timing and incidence of transmission across three years from 2010 to 2013. The results from aphid transmission, spatial spread, and post-harvest tuber incidence all imply that the $PVY^{EU\text{-}NTN}$ is the fittest strain under field conditions. It is transmitted to bait plant more effectively and it infects tubers more readily than other strains allowing for a source of inoculum to the next generation of tubers. Possible explanations for the prevalence of the $PVY^{EU\text{-}NTN}$ isolate within the trial plot include increased transmission by aphids, increased movement of the virus within the plant leading to a greater infection rate within daughter tubers and a competitive advantage when subjected to multiple isolates within the same plant. Along with this, more varieties are susceptible to PVY^{N} than PVY^{O} rouging may be more difficult if symptoms are less severe. It is not only PVY^{N} that has become predominant but $PVY^{EU\text{-}NTN}$ and our studies have successfully demonstrated that the $PVY^{EU\text{-}NTN}$ isolate outcompete $PVY^{NA\text{-}NTN}$ in all areas studied. The control of PVY is complicated by its complex epidemiology and all factors should be taken in to account when attempting to prevent the spread of the disease.

Chapter 5 - General Conclusions & Future Prospects

5.1 Conclusions

Over the past decade, PVY^{N} prevalence has been increasing in Scottish seed potatoes and now accounts for the majority of all viruses found at field inspections. In order to identify which factors were responsible for this increase and which potential threats PVY^{N} might pose to the Scottish seed potato industry, it is important to characterize the nature of PVY field isolates. Numerous studies have reported the occurrence of recombinant PVY^{NTN} strains worldwide affecting potato crops, replacing non-recombinant PVY^{O} and PVY^{N} strains (Boukhris-Bouhachem *et al.*, 2010, Chikh Ali *et al*., 2013). The results presented in Chapter 2 demonstrate that partial sequencing of recombinant junctions on the PVY genome can be a useful tool in the molecular characterisation of isolates. Using this approach for the molecular characterization of PVY, this has revealed that the vast majority of isolates (ca.75%) are clustering within the European NTN (EU-NTN) clade, demonstrating that the $PVY^{EU\text{-}NTN}$ group is widespread and prevalent in Scottish seed crops. Our results demonstrates as well that the population structure of PVY variants in Scotland is in accordance with studies that have shown recombinant isolates have become more prevalent worldwide (Revers *et al.*, 1996) and that NTN is now found in most potato growing countries (Moravec *et al.*, 2003). The distribution of PVY strains is however not uniform across the globe, as for example the N-Wilga strain is prevalent in the USA and accounts for 47% of PVY cases (S. Gray, University of Cornell, USA, personal communication) and up to 80% in Poland (Chrzanowska,

2001), while being under-represented in Scottish seed potatoes (~ 5%, C. Lacomme, SASA, personal communication).

The nomenclature of PVY strains is still a matter of debate and cannot be resolved on the basis of nucleotide sequences alone. Strain groups present were identified using Hypersensitive Response (HR) reactions in differential potato cultivars. All of the potato plants inoculated were susceptible to the limited number of PVY isolates tested, this thereby defined them as belonging to the PVY^N biotype with one isolate belonging to the PVY^E biotype. The fact that the PVY^E isolate cluster within the EU-NTN molecular group suggests that despite their related genetic background, EU-NTN isolates can elicit very diverse symptoms on their host. This illustrates the limitation of SNP analysis approaches as they may not be effective in identifying the biological characteristics of all PVY isolates.

Virus levels in Scottish seed crops are quantified through field inspections, where foliar symptoms are used to identify infected cultivars. PVY^N is known to be less symptomatic than PVY^O (Weidemann, 1988). With new recombinant strains of PVY^N now accounting for the majority of virus in Scottish seed potatoes it is important to understand what effect these isolates will have on foliar symptoms of cultivars and thereby how readily detectable they will be. Our results presented in Chapter 3 revealed that differences in foliar symptom severity can be observed between cultivars infected by the same isolate and also between isolates on the same cultivar. We demonstrated that some cultivars can be virtually asymptomatic (supporting latent infection)

while infected with PVY^N. With field inspections dependent upon the ability of trained inspectors to identify symptomatic plants, latent symptomless infections represent a risk of propagating further virus inoculum into next field generations. We would advise testing new cultivars not only for susceptibility to PVY^N but also to ensure whether a cultivar is prone to develop latent infections to PVY^N, to advise on the potential limitation of field inspections for specific cultivars and when necessary consider alternative methods such as compulsory post-harvest tuber testing for specific cultivars. Overall EU-NTN isolates produced the most severe leaf symptoms in the cultivars tested, which suggests that the dominance of this strain type in Scottish seed potatoes is unlikely to be only attributed to a lower detectability at inspection. No clear relationship could be made between leaf symptoms and tuber symptoms. Potato Tuber Necrotic Ringspot Disease (PTNRD) has been responsible for major crop losses across Europe. Factors which contribute to the development of the disease include genetic background of the host, the molecular characteristics of the virus isolate and importantly environmental conditions such as temperature (Le Romancer and Nedellec, 1997). Our results presented in Chapter 3 show that for all PVY^N isolates tested when in combination with susceptible potato cultivars, PTNRD was likely to develop under permissive conditions. PTNRD has not been reported from seed crops in Scotland and our observations in field conditions would suggest that environmental factors (including temperature) may inhibit the onset of PTNRD in Scotland. These results could have implications for export as PTNRD may develop in countries with a more favourable climate. In the highly PTNRD sensitive cultivar Nadine, plants failed to grow from affected tubers thereby limiting virus

spread. These extreme cases of severe pathogenicity represent a negative selective pressure for pathogenic PVY variants.

In order to establish efficient methods for controlling PVY, it is important to understand what is driving PVY population structure and why $PVY^{EU\text{-}NTN}$ is becoming so successful. Our results from field transmission studies presented in Chapter 4 suggest that the timing of transmission of PVY isolates is comparable indicating that the same aphid species are responsible for the majority of transmission of the three selected PVY strains, however this may vary when vector pressure is low. There are potentially three main factors that could explain EU-NTN prevalence. Firstly, there are differences in aphid transmission rates. The most efficiently transmitted strain is the $PVY^{EU\text{-}NTN}$, while significant, this parameter alone is not sufficient to explain the predominance of EU-NTN in tuber progeny. Possible explanations for increased transmission of this strain include molecular recombination events within the HC-Pro and CP coding regions of the viral genome, which might improve the acquisition of the virus to the aphid stylet. It is also possible that the virus can affect the plant chemistry in such a way to attract more aphids to probe on the plant. Secondly, EU-NTN might be more efficient in systemic movement and replication within the plant and in infecting tubers. The third factor is intraplant competition between different PVY strains. Indeed, a strong interaction was found between different PVY strains. It is possible that when more than one of PVY strains are present within a plant, direct competition for host resources are likely to occur. Our study presented in Chapter 4 suggests that the relative prevalence of $PVY^{EU\text{-}NTN}$ isolate might be caused by a combination of these factors. Rolland *et al.* (2009) found that

there was a decreased fitness of necrotic strains of PVY in tobacco compared to the non-necrotic strains. Conversely PVY^N which produce necrosis in tobacco produce fewer symptoms in potato than PVY^O and so may have an increased fitness. PVY^N is known to affect a wider range of cultivars than PVY^O, suggesting that host resistance may also play a role in the predominance of PVY^N isolates.

In order to control PVY^N in field conditions a few options are available. Results from modelling of PVY impact on crop yield indicates that the main factors are a combination of high early season vector pressure combined with tuber borne infection (Kirchner *et al.*, 2011). Planting crops early will avoid the peak of aphid virus transmission when plants are at their most susceptible developmental stage. Aphid monitoring can alert growers to the risk of transmission of the virus. Due to the non-persistent transmission of PVY by aphids, it is difficult to control PVY spread with aphicides. Mineral oils have been shown to offer a level of protection against PVY in many European countries (Weidemann, 1988). Field inspections and control of the virus through removal of symptomatic plants is still important to remove the source of inoculum from a crop. Otherwise plant breeding or transgenics may be the best way forward to control PVY (Racman *et al.*, 2001). Plants have developed a wide array of resistance mechanisms to PVY including extreme resistance, if this resistance could be introgressed into cultivars without a loss in fitness and marketability, this could help to control PVY. PVY is a rapidly evolving virus and it is important to understand its biology and how it is adapting in order to stay one step ahead in the control of this virus.

5.2 Future prospects

Following on from this project it would be valuable to include PVY^{N} ($PVY^{EU\text{-}NTN}$ variant) in resistance trials into plant breeding programmes in addition to other less prevalent molecular variants (PVY^{O}, $PVY^{N\text{-}Wilga}$, $PVY^{NA\text{-}NTN}$) as breeding resistance to one single PVY strain my not be suitable for another strain or new variant. Along with this, assessment of leaf symptom and tuber symptom development will inform classification schemes on the appropriateness of growing crop inspections for a given cultivar, and the threat of PTNRD for exports. Our experiments on leaf symptom expression were conducted in permissive glasshouse conditions, to be more informative leaf symptom expression should also be assessed in cultivars grown in the field. Further analysis of the PVY^{E} isolate 10088 may provide information on alternative locations of molecular determinants of vein necrosis in tobacco. The lack of PTNRD development from the $PVY^{N\text{-}Wilga}$ isolate on the highly susceptible cultivar Nadine demonstrates that this isolate could be used in conjunction with PVY^{NTN} isolates to identify the molecular determinants of PTNRD.

For potatoes as for other plant species, pathosystems involving viruses are highly complex. In addition to the interactions between pathogen, plant and environment, further complexity is brought in by the vectors that transmit the virus from plant to plant, environmental factors, vector species, timing of transmission, distance and control strategies used. Due to their relative plasticity of their genome, RNA viruses evolve rapidly and have the potential

to adapt (*i.e.* selection of fittest individual) to their hosts (Steinhauer and Hollland, 1987) and environmental conditions. Our studies demonstrate that $PVY^{EU\text{-}NTN}$ has been most effectively transmitted to bait plants and most effectively translocated in to tubers. Further experimentation to dissect the relative importance of intraplant movement (local and phloem-mediated), mature plant resistance (i.e. developmental stage at which infection by different PVY species still occur) and aphid-virus interactions to understand the cellular and molecular basis of $PVY^{EU\text{-}NTN}$ prevalence would be a valuable follow up to this work. Further experiments to complement this work would be to study plant preference behaviour studies on aphids feeding on inoculated and virus free plants to investigate whether PVY is able to manipulate the plant chemistry and therefore attractiveness of one strain type more successfully than another strain type. In addition, in order to evaluate the relative fitness of different PVY strains, further experiment, assessing viral movement in potato plants should be attempted. PVY moves through the phloem of the plant to infect the whole plant. Our initial attempt to assess virus movement on Maris Piper and Nadine plants failed due to low level of infectivity. Our current working hypothesis relies on the fact that if one PVY strain spread faster to infect the entire plant at a higher rate than other strains, this strain will both infect tubers more successfully and be available for aphid transmission at an earlier stage. Further analysis of competition of isolates should also be conducted preferably in field conditions as glasshouse conditions may not represent the environmental conditions required to exert a selective pressure to the most prevalent strain in the field.

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