Molecular Characterization of Potato Virus Y (PVY) and its Existing Status in Kashmir

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(2017-H-176-M)

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Dedicated

To Almighty

Allah

Sher-e-Kashmir
University of Agricultural Sciences & Technology of Kashmir
Faculty of Horticulture, Division of Plant Pathology

Certificate – I

This is to certify that the thesis entitled, **"Molecular Characterization of Potato Virus Y (PVY) and its Existing Status in Kashmir"** submitted in partial fulfilment of the requirements for the award of the degree of **Master of Science in Horticulture (Plant Pathology),** to the **Faculty of Horticulture, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir** is a record of bonafide research work carried out by **Ms. Sumiah Wani (Regd. No. 2017-H-176-M)** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that any help or information received during the course of investigation has duly been acknowledged.

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ABSTRACT

The present study entitled "Molecular Characterization of Potato Virus Y (PVY) and its Existing Status in Kashmir" was carried out during 2017-2019 at the Division of Plant Pathology, Faculty of Horticulture, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir.PVY is a widely distributed virus and ranks fifth among the top ten economically damaging viruses worldwide. This study was conducted to reveal the incidence of PVY on potato samples collected from the different growing districts of Kashmir, using biological methods (field inspections) and molecular tools were. A total of 81 potato leaf samples from inspected fields of different districts in Kashmir were collected during 2018 and 2019 growing seasons of potato. All the samples were tested by Reverse Transcription Polymerase Chain Reaction (RT-PCR) using coat protein specific primers (CP Primers). The field surveys revealed a variety of symptoms such as stunted growth, yellowish green mosaic, wilting, rugosity and general yellowing, indicating the presence of putatively viral diseases. The disease incidence was found to range from 25 to 52.38 %, with the maximum incidence recorded in Srinagar district followed by Anantnag, Baramullah and Budgam districts. Later the identification of different strains of PVY was done using strain specific primers and NTN, N and O strains of PVY were confirmed in Kashmir.

That presence of PVY in the fields of commercial potato growing areas, according to the report sounds an alarming future threat and needs action to prevent its spread. Also these results are important in providing a platform helpful for further studies to create the proper management strategies to control viral diseases of potato in Kashmir.

Key words: Potato**,** Potato virus Y, RT-PCR, Coat protein, Strain

Signature of Student
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CONTENTS

Chapter	Particulars		Page No.
1.	INTRODUCTION		1-3
2.	REVIEW OF LITERATURE		4-13
	2.1	Economic importance of PVY	5
	2.2	Symptoms of PVY	5
	2.3	Strains of PVY	6
	2.4	Host range	9
	2.5	Genome of Potato Virus Y	9
	2.6	Epidemiology	12
3.	MATERIALS AND METHODS		14-22
	3.1	Disease status	14
	3.2	Collection of samples and maintenance of virus isolates	14
	3.3	Molecular detection	16
	3.4	Sequencing of PCR product	21
	3.5	Phylogenetic analysis	22
	3.6	Screening of available germplasm	22

4.	EXPERIMENTAL FINDINGS		23-37
	4.1	Status of PVY	23
	4.2	Identification of virus	23
	4.3	PVY strain identification	26
	4.4	Molecular Characterization of PVY Strains	28
	4.5	Screening of available germplasm	36
5.	DISCUSSION		38-43
6.	SUMMARY AND CONCLUSION		44-45
	LITERATURE CITED		*i-x*

LIST OF TABLES

Table No.	Particulars	Page No.
1	Function of PVY proteins (Urcuqui-Inchima *et al.*, 2001)	11
2	Number and distribution of the locations that were surveyed for sample collection	15
3	Primer sequences of coat protein and strain specific primers of PVY	16
4	List of PCR reaction mixture components and the volume used for amplification of PVY coat protein	19
5	PCR thermal cycling regime used to amplify PVY coat protein and strain specific target regions	19
6	Pattern of Multiplex PCR for the identification of different strains	20
7	Per cent disease incidence of PVY in different locations of Kashmir based on symptomology of random selected samples	24
8	Per cent disease incidence of PVY in different locations of Kashmir based on RT- PCR detection of random selected samples	25
9	Distribution of PVY strains in different districts of Kashmir valley	27
10	Grouping of different isolates into PVY strains	27
11	Sequences of NTN, and O strains of PVY from Kashmir	29
12	Potato Virus Y isolates/strains used for accessing nucleotide Phylogenetic similarity for Coat Protein	33
13	Potato Virus Y isolates/strains used for accessing nucleotide Phylogenetic similarity for NTN Strain	34
14	PVY Isolates/strains used for accessing nucleotide Phylogenetic similarity for "O" Strain	35
15	Screening of available germplasm using Multiplex PCR with PVY, PVX, PLRV, PVS and PVA Primers	36

LIST OF FIGURES

Fig. No.	Particulars	After page No.
1.	A map showing the sampling locations in each district included in the study	14
2.	Phylogenetic analysis of the PVY-CP-Kashmir by Maximum Likelihood method. The tree depicts the relationship of PVY-CP-Kashmir with other PVY isolates at CP level	35
3.	Phylogenetic analysis of the PVY-NTN Strains by Maximum Likelihood method. The tree depicts the relationship of PVY -NTN-Kashmir Isolates with other PVY - NTN Strains throughout the world	35
4	Phylogenetic analysis of the PVY- "O" Strains by Maximum Likelihood method. The tree depicts the relationship of PVY "O" -Kashmir Isolates with other PVY "O" Strains throughout the world	35

LIST OF PLATES

Plate No.	Particulars	After page No.
1.	Symptoms of PVY observed during the field survey: a: Mosaic; b: Stunted growth; c: Leaf malformation and d: Presence of main vector (Aphids)	24
2.	Gel pictures showing; A) Isolated RNA, B) 900bp CP amplification of samples infected with PVY	25
3.	Multiplex PCR Product separated on 1% Agarose gel, Lane 1: 1kb ladder, A)NTN & O strain(452bp & 267bp respectively).B) NTN & N strain (452bp & 398bp respectively)	27

Chapter 1

INTRODUCTION

"Pray for peace and grace and spiritual food for wisdom and guidance, for all these are good. But don't forget the potatoes" (Wale *et al.,* 2008).

Potato, (*Solanum tuberosum L*), an auto tetraploid member of family *Solanaceae,* is a starchy, tuberous crop which is staple food in many parts of the world. It ranks fourth in the world in terms of production after wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.) and maize (*Zea mays* L.) (Haan and Rodriguez, 2016). The cultivated potato is the world's important food and vegetable crop containing 78% water, 18% starch, 2% protein, 1% vitamins and several trace elements (Haase, 2008). Potato is considered as the king of staple food and is recognized as a wholesome food and richest source of energy in most countries of the world (Brown, 2005). Potato is a highly recommended food security crop that can help shield low-income countries from the risks posed by rising international food prices.

As per the reports of National Horticulture Board (2017), the total area in India under potato cultivation is around 2.179 million hectares with production of about 48.605 million metric tonnes. Potato is produced in over 149 countries worldwide with more than 6 million people consuming it (Beuch, 2013). Top five potato producing countries are China, India, the Russian Federation, Ukraine and the United States. Potato plays a very important role in human diet and this has been realized by FAO and the year 2008 was celebrated as the year of potato with the slogan of the "Hidden Treasure". In recent years, there has been a shift in the end use of potatoes, with the production for direct consumption being replaced by processing potatoes for the production of convenience foods, such as french fries and potato chips.

In Kashmir cultivated potato is one of the major field crops and statistics shows that the area under potato cultivation in Jammu and Kashmir is spread over

6.54 thousand hectares with total production of 89.58 thousand tonnes (Ashwani *et al.,* 1947). It is grown as a kharief crop in Kashmir and many farmers depend on the stored tubers of previous year's kharief season. Number of farmers depends on stored tubers of spring crop to be used as seed potato for the next fall planting season.

Potato is susceptible to wide range of fungal, bacterial and viral diseases as well as various nematodes and pests. Besides *Phytopthora infestans* (the late blight pathogen), viruses are the most damaging pathogens of potato. Viruses are important pathogens that substantially reduce the yield and quality of this crop. More than 40 viruses are reported which can infect potatoes in the field and among them the most important viruses are *Potato virus Y*(PVY), *Potato leaf roll virus*(PLRV),*Potato virus X*(PVX),*Potato virus A*(PVA),*Potato virus S*(PVS) and *Potato virus M* (PVM).Other viruses occur in potato only occasionally or locally (Salazar, 2003).

Potato virus Y (PVY) is a major pathogen of potatoes cultivated worldwide and cause 80% yield loss (Quenouille *et al.*, 2013). PVY is a type member of genus *Potyvirus* in the family *Potyviridae*. The host range of PVY is mainly members of the *Solanaceae* family, although some members of *Amaranthaceae*, *Chenopodiaceae*, *Asteraceae* and *Fabaceae* are also susceptible (Kerlan, 2006). It is economically harmful to the potato industry for multiple reasons. For all growers, PVY reduces yield (Nolte *et al.*, 2004; Whitworth *et al.*, 2006). Seed growers have the additional concern of having their seed lots downgraded or deemed ineligible for recertification if PVY incidence exceeds the 0.5% certification tolerances.

The PVY viron is about 730 nm long (Delgado-Sanchez and Grogan, 1970) and 11nm wide (Varma *et al.*, 1968).The coat protein makes up about 95% of the mass of virons of Potyviruses (Hollings and Brunt, 1981). PVY has a monopartite genome composed of single-stranded positive sense RNA, approximately 10 kb in length (Makkouk and Gumpf, 1974). The genome encodes a large polyprotein that is cleaved by virus-encoded proteinases into nine or more functional proteins (Urcuqui-Inchima *et al.*, 2001).

PVY is transmitted predominantly via vegetative propagation of infected material and aphid transmission. According to Radcliffe and Ragsdale (2002), the peach aphid (*Myzus persicae*) is an important vector in transmitting PVY. Historically, PVY had three main strains: PVYO, PVYN and PVYC. PVYO is known as the common or ordinary strain. It has a worldwide distribution as per the reports of De Bokx and Huttinga (1981) and some recombinant strains are also reported. Currently, the most common recombinant strain groups in potato production are PVYN:O, PVYN-Wi and PVYNTN (Karasev and Gray, 2013).

Potato is an important cash crop of Kashmir region. Keeping in view the importance of potato crop, economic loss caused by PVY and the limited availability of studies on PVY in Kashmir, the research entitled "**Molecular Characterization of Potato Virus Y (PVY) and its existing status in Kashmir**" was planned with the following objectives:

1. Survey to document the present status of PVY in Kashmir, and
2. Identification and Molecular characterization of PVY using universal Coat Protein and strain specific primers.

Chapter 2

REVIEW OF LITERATURE

Potatoes are mainly cultivated for fresh consumption, starch production, processed foods and for seed tubers. Many characteristics are taken in consideration while selecting new potato varieties. Some of the most important traits are tuber shape, tuber skin color, tuber skin texture, flesh color, shallow eyes, yield, maturity, starch content, lack of bruising, resistance to cold, processing qualities, taste, and resistance to pathogens. It is very difficult to avoid reductions in the quality of a vegetatively propagated crop like potato when field-grown plants are used for propagation because diseases in those plants can be further multiplied each year. According to Franc (2001), the accumulation of diseases in vegetative propagated crops is known as degeneration, and the cause of degeneration is often associated with a virus. Potato is one of the oldest recorded victims of viral diseases as reported by Bawden (1964). Cultivars that are resistant to a problematic virus are critical to minimizing virus spread. One such problematic virus is potato virus Y (PVY), whose symptoms may vary depending on cultivar, strain of the virus, and even the physical environment (Draper *et al.*, 2002; Mollov and Thill, 2004).

The literature pertaining to different aspects of the present investigation has been reviewed under the following sub-heads:

2.1 Economic importance of PVY

2.2 Symptoms of PVY

2.3 Important strains of PVY

2.3.1 PVYO

2.3.2 PVYC

2.3.3 PVYN

2.3.4 PVY NTN

2.3.5 PVY N-W

2.3.6 PVY Z & E

2.4 Host range of PVY

2.5 Genome of PVY

2.6 Epidemiology of the PVY virus

2.1 Economic importance of PVY

Rolland *et al.* (2008) reported PVY among the five most economically damaging viruses. PVY in potato has received a lot of attention in recent years, and it is the most economically important disease problem in seed potatoes in many areas of the world. Potato virus Y (PVY) afflicts potato producers worldwide which results in loss of yield ranging from 10 to 80 per cent and in case of severe infection complete crop failure occurs. The extent of yield reduction depends on wide range of factors, including the viral load, the time of infection, temperature during growth and tuber storage and the cultivar of potato that is infected as reported by Warren *et al.* (2005). The most important factor which is particularly important for yield loss is the strain of PVY involved in infection. Some strains are considerably more pathogenic than others according to findings of Boonham *et al.* (2002). PVY remains viable in potato tubers. Therefore, when PVY levels are high in a seed producer's field, there is an increased initial inoculum level for next year's seed potato crop, thus increasing the chances of that year's seed lot being downgraded or rejected.

2.2 Symptoms of PVY

The development of symptoms in the host depends upon the interaction of viral proteins with molecular machinery belonging to the host plant, the level of expression of viral genes using host plant proteins and the subsequent spread of the newly formed virus particles throughout the plant (Visser, 2012). It has been revealed by the studies that plants are least susceptible when senescing but more susceptible before flowering during vegetative growth (DiFonzo *et al.*, 1994). On potato PVY shows mild to severe leaf mosaic, or streak or 'leaf-drop streak' along with vein necrosis or 'stipple-streak'. Leaves either fall from the plant or remain suspended, often giving a bare stem with leaves at the tip (De Bokx, 1972). During the primary infection by necrotic strains (PVY-N) often necrotic rings or spots develop and mild to very mild mottle late in the season occur. Secondary symptoms are usually more obvious as a severe mosaic early in the season. Some necrotic strains (PVY-NTN) cause a damaging disease with symptoms on the tubers, known as Potato tuber necrotic ring spot disease. Primary symptoms are usually present following storage, however, secondary infection may produce symptoms on harvesting (Beczner *et al.*, 1984). Infection with ordinary strain (PVY-O) causes necrosis, mottling or yellowing of leaflets, leaf drop and death. Infection with PVY-C may cause necrosis, mottling, crinkle or 'stipple-streak', and necrosis may occur on tubers. The differences between similar viruses and strains are often uncertain because of the diversity of potato cultivars and virus strains, and the effect of climatic conditions (De Bokx and Piron, 1977).

2.3 Strains of PVY

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

2.3.1 O strain (PVYO)

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

2.3.2 C strain (PVYC)

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

2.3.3 N strain (PVYN)

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

2.3.4 NTN strain (PVYNTN)

PVYNTN strain is a member of the tuber necrosis group. It was first described in Hungary in the 1980s (Ogawa *et al.*, 2008). PVYNTN is related to PVYN at a serological level. The ability to induce potato tuber necrotic ringspot disease (PTNRD) is the major characteristic that led to its differentiation from the other known strains. PTNRD is characterized by the appearance of external necrotic rings on tubers which may appear at harvest time but often develop under storage conditions. On tobacco, it causes veinal necrosis symptoms similar to PVYN (Kogovsek *et al.*, 2008; Rolland *et al.*, 2008). An important factor associated with the strain is the presence or absence of recombination. PVYNTN isolates with no recombination have been identified in North America, Denmark, Germany, Poland and Japan (Singh *et al.*, 2008). Most recombinant PVYNTN have been identified in Europe and the term Eu-PVYNTN has been used to distinguish them from the non-recombinant ones. Eu-PVYNTN generally consists of a genome that displays PVYN and PVYO like sequences, with one to three recombination junctions. The P1, HC-Pro, NIa and coat protein are the regions of the genome where recombination points were found (Kogovsek *et al.,* 2008, Lorenzen *et al.,* 2006).

2.3.5 Wilga strain

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

2.3.6 Z and E strain

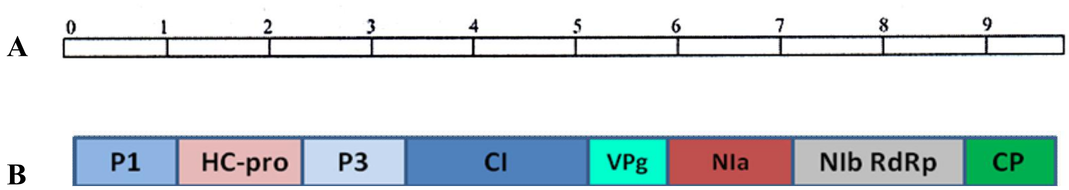
PVY Z strain (PVYZ) was proposed to distinguish the isolates serologically classified as PVYO which have overcome the resistance genes against both PVYO and PVYC but are unable to overcome the proposed *Nz* gene in the potato cultivar “Maris Bard” (Kerlan *et al.*, 1999). PVYZ has been identified in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

2.4 Host range

PVY has a wide host range, naturally infecting plants comprises up to nine families including important crops such as pepper (*Capsicum* spp.), potato (*Solanum tuberosum*), tobacco (*Nicotiana* spp.), tomato (*Lycopersicon esculentum*), some ornamental plants (*Dahlia* and *Petunia* spp.) and many weeds (*Datura* spp., *Physalis* spp., *Solanum dulcamara* and *S. nigrum*) (Jeffries, 1998). It has been reported that PVY infects natural and commonly occurring host plants and weeds such as chicory(*Cichorium intybus*), horseweed (*Conyza canadensis*), prickly lettuce (*Lactuca serriola*), dandelion (*Taraxacum officinale*), shepherd's purse (Capsella *bursa-pastoris*), common lambsquarters (*Chenopodium album*), buckhorn plantain (*Plantago lanceolata*), bittersweet nightshade (*Solanum dulcamara*) and black nightshade (*Solanum nigrum*). The experimental host range is reported to comprise 495 species in 72 genera of 31 families including 287 species in 14 genera of the Solanaceae (among which 141 Solanum species and 70 Nicotiana species), 28 species of Amaranthaceae, 25 species of Leguminosae, 20 species of Chenopodiaceae, and 11 species of Compositae (Edwardson and Christie, 1997).

2.5 Genome of Potato Virus Y

PVY was first recognized in potato by Smith (1931) as a member of a group of pathogens associated with potato degeneration, a disorder known since the 18th century. It has been one of the most studied plant viruses. Potato virus Y (PVY) is the type species of the *Potyviridae* family and the *Potyvirus* genus. This genus includes 142 approved and 32 tentative species thus making it the largest group of plant viruses (Adams *et al.,* 2012). PVY genome is composed of single-stranded positive sense RNA, approximately 10 kb in length (Makkouk and Gumpf, 1974). The nucleic acid of PVY is a single-stranded linear RNA with a sedimentation coefficient of 25S to 39S and a molecular weight of about 3.1 x 10^6 (Makkouk and Gumpf, 1974). It has a VPg cap at the 5' terminus and a polyA sequence at the 3' terminus. No sub genomic RNA is produced. The percentage of RNA in particles is from 5.4 to 6.4% (Makkouk and Gumpf, 1974; Leiser and Richter, 1978). Protein content in the particle is about 94%. Only two proteins, VPg and coat protein (CP) are detected in viral particles. CP molecular weight was calculated to be 29.95 kDa. PVY encodes a single, large polyprotein which is later processed by three virus-encoded proteinase into ten polypeptides (Figure 1) which include the following: P1, Helper component-proteinase (HC-Pro), P3, 6K1, cytoplasmic inclusion (CI), VPg, 6K2, nuclear inclusion a (NIa), nuclear inclusion b, RNA-dependant RNA polymerase (NIb-Pol) and the capsid protein (CP) (Hu *et al.*, 2009; Urcuqui-Inchima *et al.*, 2001). The functions of these proteins are summarized in Table 1.



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

Table 1: Function of PVY proteins (Urcuqui-Inchima *et al.*, 2001)

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

2.6 Epidemiology

The spread of PVY from plant to plant occurs through aphids, mechanical means or by contact. More than 50 aphid species transmit the virus in a non-persistent manner. Acquisition and inoculation involve stylet penetrations into the epidermal cell layer of the plants and occur when stylets puncture plant cell membranes, though possibility for virus to be acquired and inoculated via broken plasmodesmata is not totally excluded (Powell, 1992).Viruses transmitted in this way are also known as stylet-borne viruses. Acquisition access period (AAP), inoculation access period (IAP), latent period, and the feeding state of the vector are the different factors that influence the ability to transmit the virus. The virus AAP ranges between 5 seconds to 5 minutes. Longer periods increase transmission but AAP longer than 10 minutes results in very poor to no transmission at all. Starved aphids have been reported to transmit the virus more efficiently than non-starved. Transmission of the virus is more likely to happen during sampling probes of the vector when looking for a suitable host, since transmission does not require a latent period. There is data that indicate some of the PVY strains may be more efficiently transmitted by some aphid vectors than by others (Basky and Almasi, 2005; Cervantes and Alvarez, 2008; Fereres *et al.,* 1993), although transmission efficiency will differ among virus isolates within a strain and aphid populations within a species (Verbeek *et al.,* 2010). Once the plant foliage is inoculated by aphids, virus is translocated to tubers; although the efficiency of this translocation vary among cultivars (Gray *et al.,* 2010).

In most potato growing areas the green peach aphid (*Myzus persicae*) is clearly the most common vector with high transmission efficiency. The efficiency of transmission by other vector species is comparatively low or very low (Sigvald, 1985), but inspite of this low efficiency, some species are noteworthy vectors of PVY, among these vector species some are those that colonise potatoes such as *A. nasturtii* (in Central and Eastern Europe), *Macrosiphum euphorbiae* and *Aulacorthum solani* and other species are those that visit but rarely colonise potatoes. 22 such visiting species were quoted by and some of them (*R. padi, A. pisum, B. helichrysi, Metopolophium dirhodum, C. aegopodii*) are possibly involved in epidemics due to PVY (Weidemann, 1988).

Mechanical transmission generally occurs when an infected plant and an adjacent healthy plant are wounded by wind or human activity (such as operation from equipment in field). Mechanical seed cutting can also spread PVY, which is the reason that it is always best to sanitize seed cutting equipment before using it for another variety or seed lot (Schramm *et al.,* 2012).

Chapter-3

MATERIALS AND METHODS

3.1 Disease status

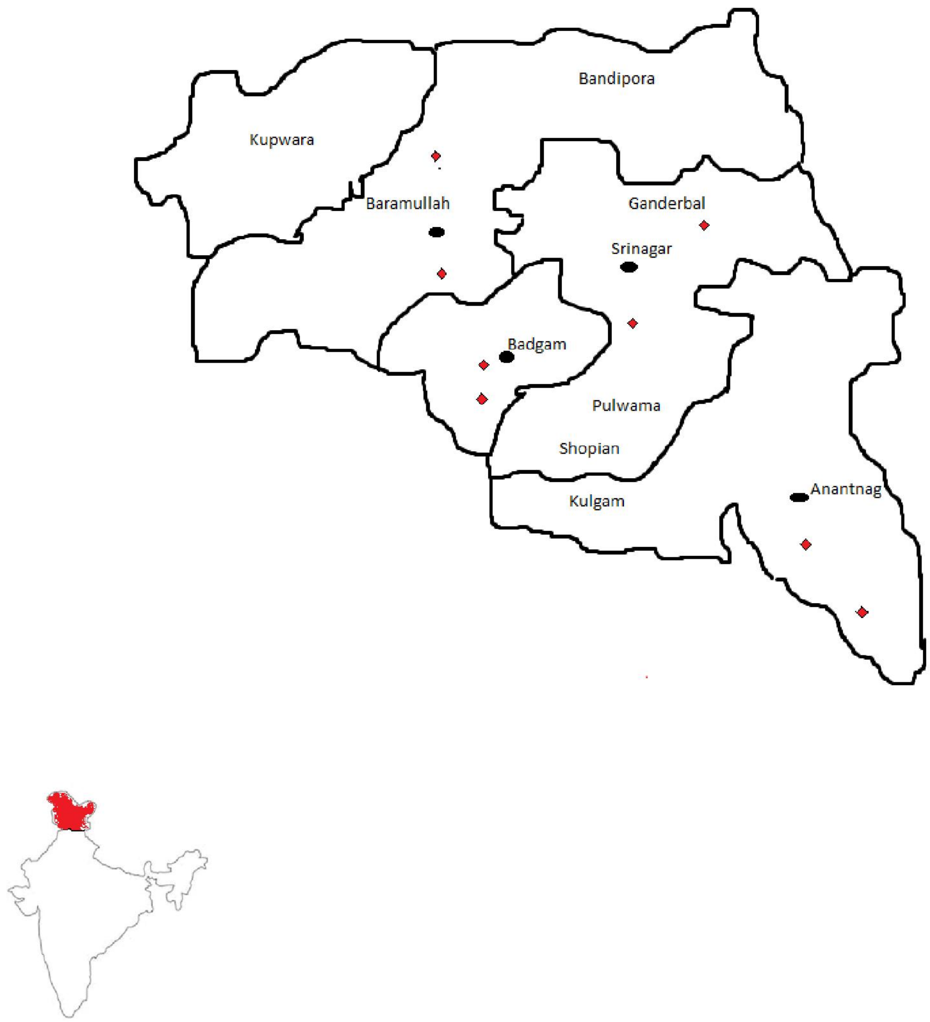
Extensive surveys of commercial and local potato growing areas of Kashmir valley were conducted during kharief 2018 and 2019 to investigate the prevalence of Potato Virus Y (PVY) disease. The diseased potato leaf samples were collected from different potato growing areas in four districts (Anantnag, Budgam, Srinagar and Baramullah) of Kashmir valley. The specific locations were Kalnag, Akingam, Mazhama, Narkara, Lal Mandi, Shalimar, Bosian and Wadura (as shown in the map). The disease incidence was recorded by counting the number of diseased plants showing PVY symptoms out of the total plants examined.

Mathematically:

$$\text{Per cent disease incidence} = \frac{\text{No. of diseased plants}}{\text{Total No. of plants examined}} \times 100$$

3.2 Collection of samples and maintenance of virus isolates

Leaf samples showing mosaic, leaf distortion and stunting were collected from infected potato plants during kharief of 2018-2019 from different locations of Kashmir valley, and the samples were categorized in 4 major groups on the basis of locality from which they were collected. Each group consisted of 20 samples which were processed in the laboratory and the isolates were maintained separately and stored at -80^{o}C for future use.



● Districts selected for survey

♦ Locations/District

Fig. 1: A map showing the sampling locations in each District included in the study

Table 2: Number and distribution of the locations that were surveyed for sample collection

S. No.	District	Block	Location	No. of samples/ Location	Total No. of samples/ District
1.	Baramulla	A) Rahama	a) Bosian	10	20
		B) Zaingeer	a) Wadura	10	
2.	Srinagar	A) Srinagar	a) Shalimar	10	20
			b) Directorate of Agriculture (Lal Mandi)	10	
3.	Budgam	A) Narbal	a) Mazhama	10	20
		B) Budgam	a) Narkara	10	
4.	Anantnag	A) Larnoo	a) Kalnag (Kokernag)	10	20
		B) Achabal	b) Akingam	10	

3.3 Molecular detection

3.3.1 Primer designing

Primer designing of coat protein gene for RT-PCR assay was done using National Centre for Biotechnology Information (NCBI) primer blast tool (https://www.ncbi.nlm.nih.gov).The downloaded sequences in the FASTA format were uploaded in Primer3 (www.https://primer3plus.com) to design the primers and for strain identification strain specific primers already designed by Lorenzen *et al.* in 2006 were used.

Table 3: Primer sequences of coat protein and strain specific primers of PVY

Primer	Sequence	Target Strain	Size (bp)
Primer 1	Forward: CAACTATGATGGATTTGGCGACC Reverse: CCCAAGTTCAGGGCATGCAT	O	267
Primer 2	Forward: GTCGATCACGAAACGCAGACAT Reverse: TGATCCACAACTTCACCGCTAACT	N	398
Primer 3	Forward: GGATCTCAAGTTGAAGGGGAC Reverse: CTTGCGGACATCACTAAAGCG	NTN	452
Primer 4	Forward: GGATCTCAAGTTGAAGGGGAC Reverse: CTCCTGTGCTGGTATGTCCT	N: O	452
Primer 5	Forward: TATGAATTCCACCATCAAGCAAATGA Reverse: ATCGTCCGGCTCGAGACTACATCA	CP	900

3.3.2 Extraction of total RNA

1) ***Total RNA isolation using TRIzol with slight modifications.***

i) Leaf tissue samples were taken out from – 80^{o}C and quickly freezed in liquid nitrogen.

ii) The frozen samples (50-100mg) were put in a chilled sterile mortar and pestle with liquid nitrogen and grounded well into a fine powder.

iii) The leaf powder was transferred in to a 1-1.5 ml polypropylene tube using a sterile spatula.

iv) TRIzol® (Life Technologies, Grand Island, NY, USA) reagent was added to the tube at the rate of 1ml per 50-100mg of tissue. The tissue was mixed until the powder suspended.

v) The sample tubes were placed on a rocking platform and shook gently for at least 5 min at room temperature.

vi) Chloroform was added @ 200 µl per ml of TRIzol.

vii) Incubated at room temperature for 2-3 minutes until the phases began to separate and the samples were centrifuged for 15 min at 12,000g at 4^{o}C.

viii) The supernatant was transferred to a new 1-1.5 ml tube without transferring any portion from the lower phase.

ix) 0.5 volume of isopropyl alcohol was added to the aqueous phase and the tubes were gently shaken for 10 minutes in a gel rocker.

x) The tubes were centrifuged for 10 minutes at 12,000g at 4^{o}C and after centrifugation the supernatant was decanted.

xi) The pellet was washed with 70% ethanol and centrifuged at 12,000g for 5 min at 4^{o}C. This step was repeated two times and then pellet was resuspended.

xii) After through washing the pellet was resuspended in RNase-free water, kept for dissolving overnight at 4^{o}C and stored at -20^{o}C till further use.

xiii) The RNA was transferred to a microfuge tube and stored at -20 ^{o}C.

2) RNA check

Purity of isolated RNA was checked by electrophoresis and by measuring A260/280 ratio in UV spectrophotometer. To check the quality of extracted RNA 5 micro litres of RNA was mixed with 2 micro litres of gel loading dye and loaded on one per cent agarose gel containing 2 micro litres of ethidium bromide and electrophoresed in 1X TAE buffer at 80 volts for 1hours. Then the gel was viewed under Gel documentation system using UV transilluminator and the image captured. The quality of RNA was checked by loading two micro litres of RNA on bio- spectrophotometer.

3) First strand cDNA synthesis

Total RNA was used for complementary DNA synthesis, using SuperScript™ III reverse transcriptase. Reverse transcription was performed in 0.2ml thin walled tubes using the total RNA as template and for reverse transcription (RT) reaction 13 micro litres of the reaction mixture (1µl of RNA+1 µl of primer reverse + 1µl of 10mM dNTP + 10µl water) was kept at 65^{o}C for 5 minutes and then snow chilled and mixed with 4µl of 5X buffer, 1µl of 0.1M DDT, 1µl of RNase inhibitor and 1µl of RT enzyme to a final volume of 20µl. The reaction mixture was incubated at 50^{o}C for1 hr. The reverse transcriptase enzyme activity was inactivated by incubating the mixture at 70^{o}C for 15 minutes in an automated thermocycler.

4) Reverse Transcription RT-PCR

RT-PCR was done using PVY universal CP (coat protein) and strain specific primers for virus and strain identification respectively. PCR was carried out with 20 µl of total reaction mixture containing 2µl cDNA product, 1µl of both reverse and the forward coat protein (CP) primer, 4µl of HF buffer, 0.4µl of 10mM dNTPs and 0.3µl of Phusion DNA polymerase. It was then subjected to PCR amplification with the following patterns.

Table 4: **List of PCR reaction mixture components and the volume used for amplification of PVY coat protein**

Components	Volume (1x)
5x Phusion HF Buffer	4.0µL
dNTPs	0.4µL
Forward Primer (10mM)	1.0µL
Reverse Primer (10Mm)	1.0µL
Phusion	0.3µL
Autoclaved Millipore water	11.3µL
cDNA	2.0 µL
Total Reaction Volume	20.0µL

Table 5: **PCR thermal cycling regime used to amplify PVY coat protein and strain specific target regions**

Step	Temperature (^{o}C)	Time
Initial denaturation	98	30 seconds
25-35 cycles	98	5-10 seconds
	45-72	10-30 seconds
	72	15-30 seconds
Final extension	72	5-10 minutes

3.3.3 Agarose gel electrophoresis of the PCR product (amplicon)

The gel electrophoresis was carried out using a submarine horizontal agarose slab gel. Appropriate amount of agarose (1%) was dissolved in 1X TAE buffer by boiling (so as to dissolve completely). This molten agarose was cooled to 50^{o}C and after adding 2µl ethidium bromide was casted in an appropriate gel casting tray using a slot forming comb. PCR products were properly mixed with 2µl of gel loading dye and loaded onto the wells of the gel. DNA ladder was also loaded to compare the size of the PCR product. Electrophoresis was carried out at 80 volts for 1 hour. After electrophoresis, the gel was visualized using a UV transilluminator and photographed using Gel Documentation system.

3.3.4 Multiplex PCR

Multiplex PCR was used to identify different strains of PVY. The reaction was carried out with 20 micro litres of total volume containing 2 micro litres of cDNA product, 0.5µl of all the ten primers (as shown below in Table 6), 0.3µl of Phusion DNA polymerase and the mixture was subjected to PCR amplification with the below given pattern.

Table 6: Pattern of Multiplex PCR for the identification of different strains

Component	**Volume (1x)**
Buffer	4.0 µL
dNTPs	0.4 µL
Forward primers (CPF,1F, 2F, 3F, 4F)	0.5×5 = 2.5 µL
Reverse primers (CPR, 1R, 2R, 3R, 4R)	0.5×5 = 2.5 µL
Phusion	0.3 µL
cDNA	2.0 µL
Autoclaved Millipore water	8.3 µL
Total volume of the reaction	20.0 µL

3.3.5 RT PCR for aphids

The aphids were collected from potato crop at experimental fields of Division of Vegetable Science, SKUAST-K, Shalimar during kharief 2019 and total RNA from aphids (about 150 adults) was isolated using TRIzol according to the protocol given above and RNA concentrations were measured by a NanoDrop. Later on cDNA was synthesized using SuperScript™ III Reverse Transcriptase. RT PCR was conducted with thermal conditions: initial denaturation at 95 ^{o}C for 3min, followed by 50 main cycles (95^{o}C for 10 seconds, 55 ^{o}C for 10 seconds, 72^{o}C for 20 seconds) and finally 1 cycle of single extension (72^{o}C for 1 minutes.).

3.3.6 Elution of the PCR amplified DNA fragment from agarose gel

The PCR product was resolved on 1% agarose gel. Strain specific bands were cut and eluted from the gel using QIAquick® Gel extraction kit and QIAquick® PCR & Gel Cleanup kit, following the manufacturer's instructions.

3.4 Sequencing of PCR product

Some selected freeze-dried purified PCR product of different strains was directly submitted for custom sequencing through custom sequence services (Life Technologies India Pvt. Ltd.) The specific primers used for amplification of target sequence of the test strains were used for sequencing.

3.4.1 Nucleotide sequence analysis

The sequence of PVY (Ant4 NTN, SL1NTN, SL3NTN, Bar4, Bar8, Ant4O, SL1O and SL3O) strains obtained were blasted using Blastn program from the website http://www.ncbi.gov/blast (Altschul *et al.*, 1997) and it was checked with the available international sequence databases. Subsequently, 15 whole genome NTN sequences and 21 whole genome O sequences were selected for sequence comparison. These multiple sequences were aligned by ClustalW program from the website http://www.ebi.ac.uk/clustalW/ (Higgins *et al.*, 1994).

3.5 Phylogenetic analysis

Phylogenetic analysis was carried out comparing the nucleotide sequences of coat protein as well as strain specific genes. Analysis was performed with maximum likelihood method using cluster W program (Higgins *et al.*, 1994) with the help of software's (https://www.megasoftware.net/, www.mbio.ncsu.edu/bioedit/bioedit.html) and Phylogenetic tree was constructed.

3.6 Screening of available germplasm

Potato seed tubers of different varieties like Shalimar Potato-1, Shalimar Potato-2, Kufri Badshah, Kufri Chipsona-1, Kufri Chipsona-2,Kufri Himalini, Kufri Girdari,P-14 etc were provided by the division of Vegetable Science (SKUAST-K, Shalimar).Potato seeds were also collected from farmer's field during the survey. The tubers were planted in pots and grown under greenhouse conditions during kharief 2019 at the Faculty of Horticulture, SKUAST-K, Shalimar. Leaf samples were collected during the vegetation period before harvest from all the plants. Samples were kept into plastic bag and storing in a freezer at -20^{o}C, later total RNA was isolated and cDNA synthesized by Superscript III. Using the method of Multiplex PCR with thermal conditions: initial denaturation at 95^{o}C for 3 minutes, followed by 50 main cycles (95^{o}C for 10 seconds, 55^{o}C for 10 seconds, 72^{o}C for 20 seconds) and finally 1 cycle of single extension (72 ^{o}C for 1minute), the samples were analysed. In addition to PVY, the primers for PVX, PLRV, PVS and PVA were used for this multiplex PCR.

Chapter- 4

EXPERIMENTAL FINDINGS

4.1 Status of PVY

Extensive surveys of commercial potato growing areas of Kashmir valley were conducted during kharief 2018 and 2019 to ascertain the status of Potato Virus Y disease. During the periodic survey it was observed that the potato growing farmers plant locally adapted varieties usually mixing different varieties together. However, the commercial potato growing centres in the Kashmir valley plant commercial cultivars such as Kufri Girdari, Himalayan and Kufri Jyoti. During the survey a variety of virus symptoms were observed in the potato fields (Plate 1). Observed symptoms included yellowish green mosaic, stunted growth, leaf malformation, yellowing and wilting. In many fields the presence of main vector of PVY (Aphid, *Myzus persicae*) was also observed. On the basis of above symptoms the disease incidence varied among the different surveyed locations.Maximum incidence being in the Shalimar area of district Srinagar (27.33) and minimum in the Kalnag area of district Anantnag. As shown in (table 7).

4.2 Identification of virus

Confirmation of the viral etiology of each isolate was done by using molecular method i.e. RT-PCR amplification of coat protein gene of the virus.

4.2.1 Molecular detection of PVY

RNA extracted from the samples was intact with RNA concentration ranging from 700ng to 2300ng (Plate 2A). Out of 81 samples collected from four different locations 31 tested positive for PVY RT-PCR method (Table 8). All of these samples were collected from the potato plants showing PVY symptoms. The specific bands of about ~900bp were obtained from infected samples when subjected to RT-PCR (Plate 2B).

Table 7: Per cent disease incidence of PVY in different locations of Kashmir based on symptomology of random selected samples

District	Location	Field	No. of plants observed	No. of plants showing PVY symptoms	Incidence/ field (%)	Average incidence of location (%)
Anantnag	Kalnag	F1	250	46	18.40	17.2
		F2	250	60	24.00	
		F3	250	23	9.20	
	Akingam	F1	250	39	15.60	16.8
		F2	250	64	25.60	
		F3	250	23	9.20	
Srinagar	Shalimar	F1	250	48	19.20	27.33
		F2	250	77	30.80	
		F3	250	80	32.00	
	Lal Mandi	F1	250	37	14.80	19.33
		F2	250	33	13.20	
		F3	250	75	30.00	
Budgam	Narkara	F1	250	26	10.40	17.86
		F2	250	48	19.20	
		F3	250	60	24.00	
	Mazhama	F1	250	14	5.60	16.00
		F2	250	36	14.40	
		F3	250	70	28.00	
Baramulla	Bosian	F1	250	78	31.20	17.33
		F2	250	21	8.40	
		F3	250	31	12.40	
	Wadura	F1	250	45	18.00	25.2
		F2	250	80	32.00	
		F3	250	64	25.60	

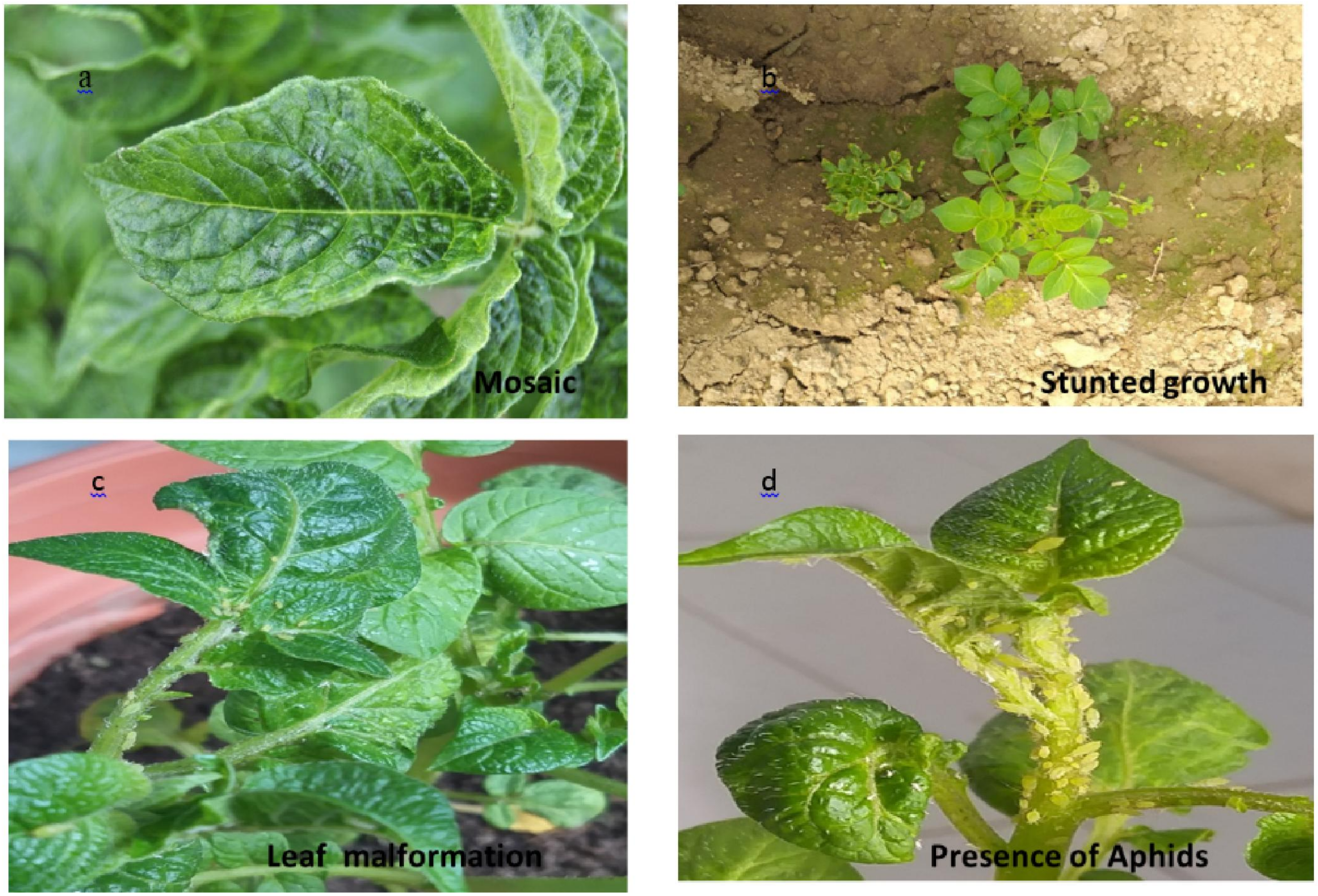


Plate 1: Symptoms of PVY observed during the field survey: a: Mosaic; b: Stunted growth; c: Leaf malformation and d: Presence of main vector (Aphids)

Table 8: **Per cent disease incidence of PVY in different locations of Kashmir based on RT- PCR detection of random selected samples**

District	Location	No. of samples tested	No. of samples infected	Incidence (%)	Mean incidence (%)
Baramulla	Bosian	10	4	40.00	35
	Wadura	10	3	30.00	
Srinagar	SKUAST-K Shalimar	14	8	57.14	52.38
	Lal Mandi	7	3	42.86	
Budgam	Mazhama	10	3	30.00	25
	Narkara	10	2	20.00	
Anantnag	Kalnag	10	3	30.00	40
	Akingam	10	5	50.00	

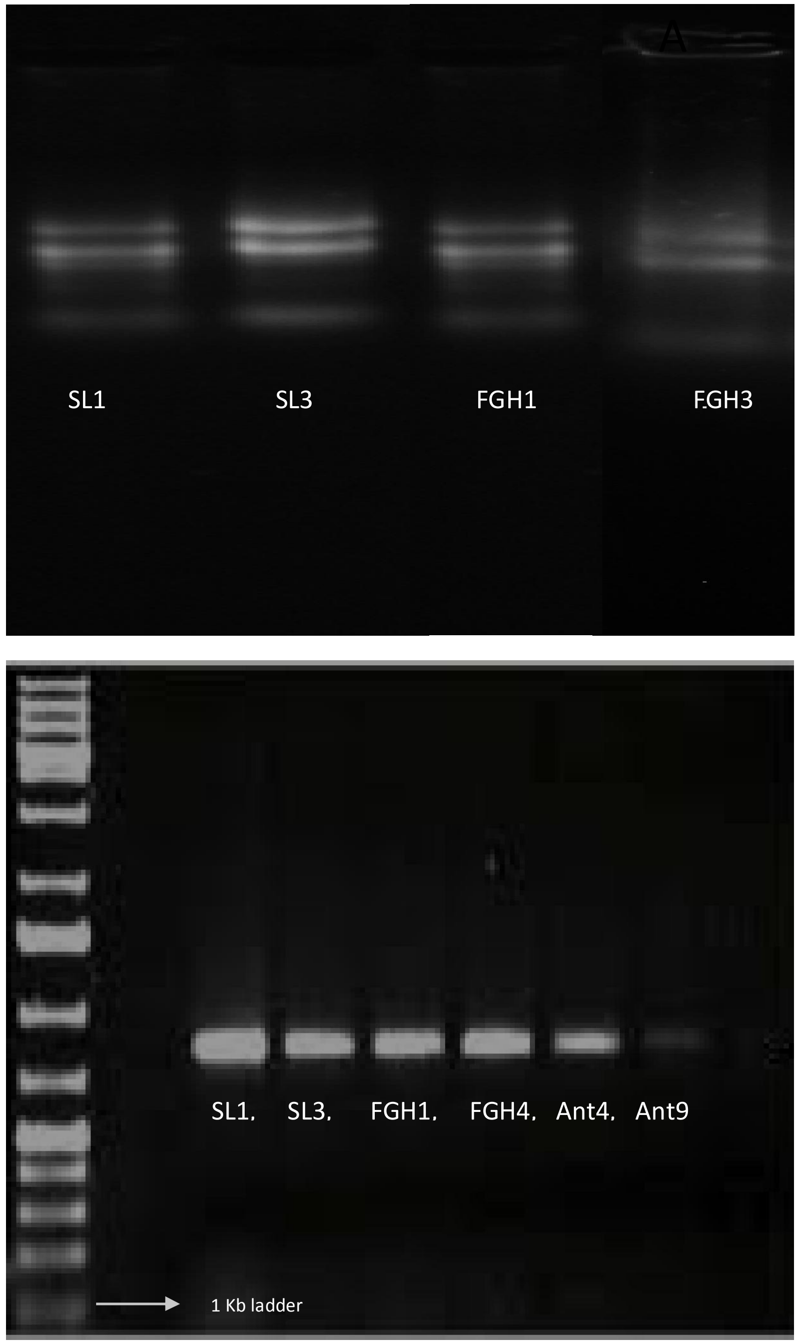


Plate 2: **Gel pictures showing; A) Isolated RNA, B) 900bp CP amplification of samples infected with PVY**

Thirty one PVY positive samples were used to calculate the actual per cent incidence in different locations (Table 8). All the surveyed locations showed presence of PVY disease with different proportion of incidence that ranged between 25.00 to 52.38 per cent. Maximum disease incidence was recorded in district Srinagar (52.38%) followed by that in Anantnag (40%), Baramulla (35%) and Budgam (25%) districts. From this data, it is concluded that there is a considerable difference in the level of disease incidence within districts as well. The percent disease incidence at two locations of district Srinagar ranged between 42.86 per cent in Lal Mandi to 57.14 per cent in SKUAST-K Shalimar. Among the two locations surveyed in district Anantnag 30.00 per cent disease incidence was observed at Kalnag whereas 50 per cent incidence was recorded at Akingam locality of Achabal. Similarly, In Baramullah district, disease incidence in different localities varied from 30 per cent (Wadura) to 40 per cent in Bosian area. Out of the four locations surveyed in district Budgam, disease incidence varied from 20.00 per cent in Narkara to 30.00 per cent in Mazhama area. The overall disease recorded was less in this district.

4.3 PVY strain identification

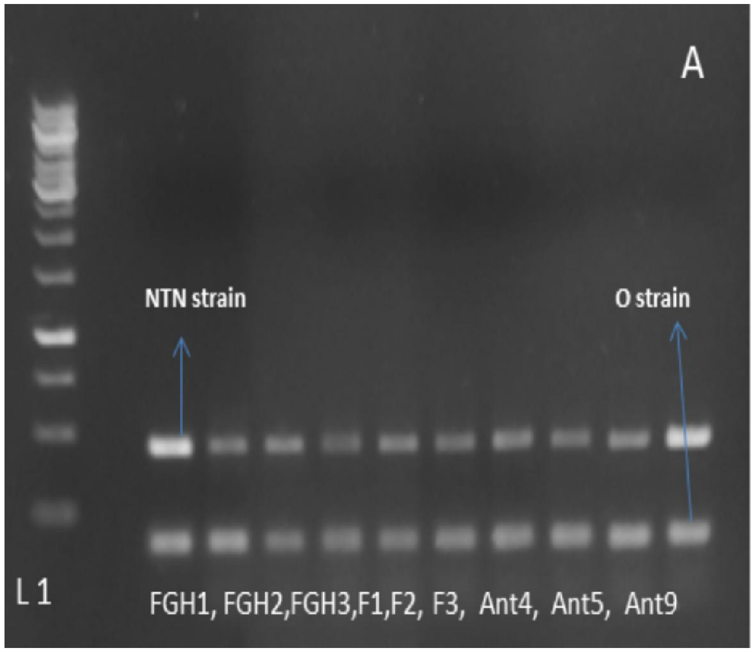
Four primer sets were used to identify PVY strains in the 31 infected samples. Of the 31 PVY infected samples identified using RT-PCR, 25 samples reacted positively with primer specific to PVYNTN, 21 samples reacted with primer specific to PVYO and 16 samples were PVYN (Table 9). And most of the isolates showed mixed infection as shown in Plate 3.

Table 9: Distribution of PVY strains in different Districts of Kashmir valley

Location	Positive Samples (On the basis of CP primers)	Strain Type (On the basis of strain specific primers by using Multiplex PCR)			
		NTN	O	N	N:O
Baramullah	**7**	5	5	4	0
Srinagar	**11**	9	7	5	0
Budgam	**5**	3	1	2	0
Anantnag	**8**	8	8	5	0

Table 10: Grouping of different isolates into PVY strains

Districts	PVY Isolate	Strains
Baramullah	Bar1, Bar2, Bar3, Bar4, Bar5	NTN
	Bar1, Bar2, Bar3, Bar4, Bar5	O
	Bar1, Bar3, Bar6, Bar7	N
Srinagar	SL1, SL3, Sha1, Sha2, Sha3, FGH1, FGH2, FGH3, FGH4	NTN
	SL1, SL3, Sha4, FGH1, FGH2, FGH3, FGH4	O
	SL1, SL2, Sha1, Sha3, FGH2	N
Budgam	SS1, SS2, SS6	NTN
	SS1, SS3	O
	SS11	N
Anantnag	Ant3, Ant4, Ant5, Ant6, Ant9, Ant11, K1, K10	NTN
	Ant3, Ant4, Ant5, Ant6, Ant9, Ant11, K1, K10	O
	Ant5, Ant6, Ant8, Ant9, Ant11	N



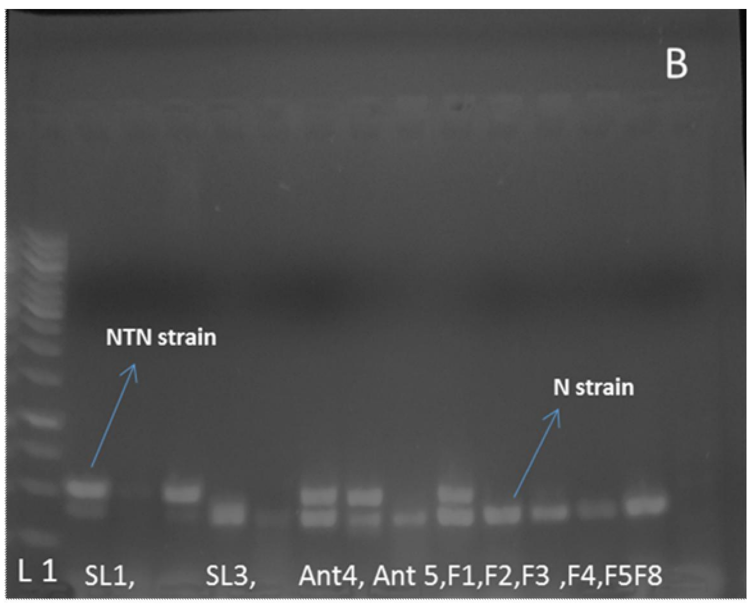


Plate 3: **Multiplex PCR Product separated on 1% Agarose gel, Lane 1: 1kb ladder, A)NTN & O strain(452bp & 267bp respectively).B) NTN & N strain (452bp & 398bp respectively)**

4.4 Molecular Characterization of PVY Strains

The PVY strains described above were characterized on the basis of strain specific primers to ascertain their variability at molecular levels.

4.4.1 RT-PCR amplification of Coat Protein gene and strain specific genes

Total RNA was isolated from the selected leaf samples infected with PVY by using TRIzol as per manufacturer's instructions, and checked on 1.0 per cent agarose gel for optimum concentration of genomic RNA. cDNA strand was synthesized using isolated RNA with oligo dt/CP downstream primer by reverse transcription (Superscript reverse transcriptase enzyme) and PCR amplification of both coat protein gene and strain specific genes was done using upstream and downstream specific primers in the different selected samples. Gel electrophoresis of the amplified RT-PCR product yielded a predicted band of ~900 bp for CP and ~452 bp(NTN), ~398bp (N) and ~ 267 bp(O) on 1% agarose gel.

PCR product (DNA) was eluted from the gel using DNA Gel Extraction Kit (QIAquick® Gel extraction kit and QIAquick® PCR & Gel Cleanup kit) as per manufacturer's instruction and was checked on 1.2 per cent agarose gel.

The amplified PCR product of aphid RNA yielded a band size of ~452 bp indicating the presence of NTN strain in the isolate.

4.4.2 Sequencing of PCR-amplified product

Purified PCR product of CP(~900bp), NTN (~452bp), N(~398bp) and O (~267bp) strains was send for custom sequencing along with upstream and downstream strain specific primer. The PCR product sequenced through custom sequence services (sequencing @geneilabs.com) revealed that the test sequences obtained through upstream and downstream strain specific primers in automated sequencer consisted of CP(~900bp), ~452 bp(NTN), ~398bp (N) and ~ 267 bp(O) and the conting was developed using bio edit software, given below in Table 11.

Table 11: Sequences of NTN, and O strains of PVY from Kashmir

>ANT4NTN

CAGCTAGACGTTCTCTTCAATTTGAGCTCCAGTGAGCGGATCAACGAA
CTGGATGAACGAATATTCTGTTGGGTCAAATCCATACATATTAACAAA
CCTCCTGCTTGACTTGCCCATACCAACAGTGGTGCCTTTACCTTTTCCC
TTCTTCCTGTATACAGATTCTCAATAGAATTCCTCTATTGTGTCATCAT
ATGTTGAAAAATCTCAAAGCCAACCATTTTGTCACGAGCATGGCGAAA
CTTCAAGGCTTGAATTCTTTTGGATTTATTTTTGCCCTTGGTGAGACAC
AGTCTCAACTGATTGTGTCAATCCAACTATATATGAGTCCTATTCCACC
AATTGCAACAGCGCCTGCTATGATCAAGTCTTTGGCCACTAATGACTT
CTTCCAAGTCCCCTTCAACTTGAGATCCAGA

>Bar4NTN

GCCAAAACACGACCGCAAAAGAGAACACCACGCTTTCTACTCGCTGAC
ATCACTAAAGCGCTCTTGGATGTCTCTAATATCAGCATAGAACGTTCTC
TTCAATCTTGAGCTCCAGTGAGCGGATCAACGAACTGGATGAATGAAT
ATTCTGTTGGGTCAAATCCATACATATTAACAAACCTCCTGCTTGACTT
GCCCATACCAACAGTGGTGCCTTTACCTTTTCCCTTCTTCCTGTATGCA
GATCCAAAGAATTCCTCTATTGTGTCATCATTGTTGTCAATTTCAAAGC
CAGCCCTTTTGTCACGAGCATGGCGAAACTTCAAGGCTTGAATTCTTTT
GGATTTATTTTTCCCTTGGTGAGACACAGTCTCAACTGATTGTGTGAAC
CAACTATATATGAGTCCTATTCCACCAATTGCTAACAGCCGCTCTGCTA
AGATCAAGTCTTGGCCACTAATGACTTCTTCCAAGTCCCCTTCAACTTG
AGATCCAAAGA

>Bar8NTN

GTTAAGCTTACCTTCCGCTTTTATGATGTCCGCAATCCTCCTCTTTGTTG
ATCCGATCGCTGGATCTCAAGTTGAAGGGGACCACTTCGCTTTAGTGA
TGTCCGCAAGCATCCAGATTGGTTGATCCGACTCACTTTCTTCTTGCGG
ACATCACTAAAGCGCTCTTGGATGTCTCTAATATCAGCATAGCACGTT
CTCTTCAATTTGAGCTCCAGTGAGCGGATCAACGAACTGGATGAACGA
ATATTCTGTTGGGTCAAATCCATACATATTAACAAACCTCCTGCTTGAC
TTGCCCATACCAACAGTGGTGCCTTTACCTTTTCCCTTCTTCCTGTATGC
AGATCCAAAGAATTCCTCTATTGTGTCATCATTGTTGTCAATTTCAAAG
CCAGCCCTTTTGTCACGAGCATGGCGAAACTTCAAGGCTTGAATTCTTT
TGGATTTATTTTTCCCTTGGTGAGACACAGTCTCAACTGATTGTGTGAA
CCAACTATATATGAGTCCTATTCCACCAATTGCAACAGCGCTCTAGCTT
AATGATCAAGTCTGTTGGCCACTAATGACTTCTTCCAAGTCCCCTTCTA
CTTGAGATCCAAAAAGTGAGCGGATCAACGAACTGGATGCTTGAGAA
AAAAAGTAACAAGAATTTATTAATGTTAAAAATAAAAATAGTTAATTA
ACTATACA

>SL1NTN

TTAACGTTCTCTTCAATTTTGAGCTCCAGTGAGCGGATCAACGAACTG
GATGAATGAATATTCTGTTGGGTCAAATCCATACATATTAACAAACCT
CCTGCTTGACTTGCCCATACCAACAGGGGTGCCTTTACCTTTTCCCTTC
TTCCTGTATGCAGATCCAAAGAATTCCTCTATTGTGTCATCATTGTTGT
CAATTTCAAAGCCAGCCCTTTTGTCACGAGCATGGCGAAACTTCAAGG
CTTGAATTCTTTTGGATTTATTTAATAAGCGCCTTGGTGAGACACAGTC
TCAACTGATTGTGTGAACAAACGTATATATGAGTCCTATTCCACCAATT
GCAACAGCCCTCAGCTATGATCAATGTCTTTGGCCACTAAAGACTTCTT
CCAATTCCTCTTCTACTTGAGAAT

>SL3NTN

AATAGGACGTTCTCTTCAATTTGAGCTCCAGTGAGCGGATCAACGAAC
TGGATGAATGAATATTCTGTTGGGTCAAATCCATACATATTAACAAAC
CTCCTGCTTGACTTGCCCATACCAACAGGGGTGCCTTTACCTTTTCCCT
TCTTCCTGTATGCAGATCCAAAGAATTCCTCTATTGTGTCATCATTGTT
GTCAATTTCAAAGCCAGCCCTTTTGTCACGAGCATGGCGAAACTTCAA
GGCTTGAATTCTTTTGGATTTATTTTTCCCTTGGTGAGACACAATCTCA
ACTGATAGAGAGAACCAACTATATAAGAGTCCTATTCAACCAATAGTA
CA

>ANT4O

TCTCAACTATGATGGATTTGGCGACAACTTGTGCTCAAATGAAAATAT
TCTACCCTGATGTTCATGATGCAGAACTGCCTAGAATACTAGTCGATC
ACGAAACGCAGACATGCCATGTGGTTGACTCGTTTGGCTCACAAACAA
CTGGGTATCATATTTTGAAAGCATCTAGCGTGTCCCAACTTATTTTGTT
TGCTAATGATGAGTTGCGAGTCTGACGGTTA

>SL1O

CCAAAGTTCAGGGCATGCTTTTCCCCAAGTTCAGGGCATGCATTAGGA
ATACCACCAACTCTATAGATGCTTAATGTCAGACTCCAACTCATCATTA
GCAAACAAAATAAGTTGGGACACGCTAGATGCTTTCAAAATATGATAC
CCAGTTGTTTGTGAGCCAAACGAGTCAACCACATGGCATGTCTGCGTT
TCGTGATCGACTAGTATTCTAGGCAGTTCTTGCATCATGAACATGCAG
GGTAGAATATTTTCATTTGAGCACAAGTTGTCGCCAAATCCATCATAG
TTGGGA

>SL3O

GGGCATGCTTTTCCCCAAGTTCAGGGCATGCATTAGGAATACCACCAA
CTCTATAGTGCTTAATGTCAGACTCCAACTCATCATTAGCAAACAAAA
TAAGTTGGGACACGCTAGATGCTTTCAAAATATGATACCCAGTTGTTT
GTGAGCCAAACGAGTCAACCACATGGCATGTCTGCGTTTCGTGATCGA
CTAGTATTACTAGGCAGTTCTGCATCATGAACATGCAGGGTAGAATAT
TTTCATTTGAGCACAAGTTGTCGCCAAATCCATCATAGTTGGG

>CP full Kashmir

GCAAATGACACAATCGATGCAGGAGGAAGCACTAAGAAGGATGCAAA
ACAAGAGCAAGGTAGCATTCAACCAAATCTCAACAAGGAAAAGGAAA
AGGACGTGAATGTTGGAACATCTGGAACTCATACTGTGCCACGAATTA
AAGCTATCACGTCCAAAATGAGAATGCCCAAGAGTAAAGGTGCAACT
GTACTAAATTTGGAACACTTACTCGAGTATGCTCCACAGCAAATTGAC
ATCTCAAATACTCGAGCAACTCAATCACAGTTTGATACGTGGTATGAA
GCGGTACAACTTGCATACGACATAGGAGAAACTGAAATGCCAACTGT
GATGAATGGGCTTATGGTTTGGTGCATTGAAAATGGAACCTCGCCAAA
CATCAACGGAGTTTGGGTTATGATGGATGGAGATGAACAAGTCGAATA
CCCACTGAAACCAATCGTTGAGAATGCAAAACCAACACTTAGGCAAAT
CATGGCACATTTCTCAGATGTTGCAGAAGCGTATATAGAAATGCGCAA
CAAAAAGGAACCATATATGCCACGATATGGTTTAGTTCGTAATCTGCG
CGATGGAAGTTTGGCTCGCTATGCTTTTGACTTTTATGAGGTCACATCA
CGAACACCAGTGAGGGCTAGGGAAGCGCACATTCAAATGAAGGCCGC
AGCATTGAAATCAGCCCAACCTCGACTTTTCGGGTTGGACGGTGGCAT
CAGTACACAAGAGGAGAACACAGAGAGGCACACCACCGAGGATGTCT
CTCCAAGTATGCATACTCTACTTGGAGTCAAGAACATG

4.4.3 Phylogenetic Analysis of CP Gene of PVY Isolate from Kashmir and Worldwide Isolates

Analysis revealed that coat protein gene of PVY isolate from Kashmir (PVY-CP Kashmir) shared identity ranged from 99.6-88.7% at nucleotide level with 19 worldwide PVY isolates used in this study (Table 12). The Maximum likelihood trees was constructed and PVY-CP Kashmir shared the highest identity at nucleotide level with PVY-CP from Hungry and Vietnam, while shared the least identity with PVY- NTN strain from Japan in case (Fig. 4).

4.4.4 Phylogenetic Analysis of strain specific genes of PVY Isolates from Kashmir and Worldwide Isolates

Phylogenetic analysis of nucleotide sequences of the strain specific genes of PVY-NTN and PVY O Kashmir isolate and 35 (16 NTN and 19 o isolates) PVY worldwide isolates was conducted using tools implemented in Mega 7 program (Table 13 & 14). The Maximum likelihood trees were constructed from all these PVY isolates. And the Phylogenetic tree of PVY NTN isolates formed two groups with PVY NTN isolates of Kashmir sharing highest similarity with PVY - NTN isolates from Brazil, China and Switzerland (Fig. 5). Whereas, PVY-O isolates formed three groups, with PVY -O isolates from Kashmir showing close proximity to PVY-O from China and Brazil (Fig. 6).

Table 12: Potato Virus Y isolates/strains used for accessing nucleotide Phylogenetic similarity for Coat Protein

Location	Accession No.	Isolate/Strain
Kashmir	NA	CP Full Kashmir
Hungary	X54611	Coat protein gene
Vietnam	DQ925437	Polyprotein gene
Iran	LN880858	CP
Brazil	JQ924287	NTN
USA	FJ204166	NTN
	EF026075	NTN
China	HQ631374	NTN
Slovenia	KM396648	NTN
Poland	KX356069	NTN
South Korea	MH603863	NTN
UK	KC634009	NTN
	KC634007	NTN
	KC634008	NTN
Brazil	JF928460	NTN
Switzerland	MF405303	NTN
	MF422609	NTN
Serbia	KJ946936	NTN
Japan	AB711146	NTN
	AB711145	NTN
	AB711143	NTN

Table 13: Potato Virus Y isolates/strains used for accessing nucleotide Phylogenetic similarity for NTN Strain

Location	Accession No.	Isolate/Strain
Kashmir	NA	Ant4 NTN
	NA	Bar4NTN
	NA	Bar8NTN
	NA	SL1NTN
	NA	SL3NTN
Brazil	JQ924287	NTN
	JF928460	NTN
USA	FJ204166	NTN
	EF026075	NTN
	AY884982	NTN
China	HQ631374	NTN
South Korea	MH603863	NTN
	MH603863	NTN
UK	KC634009	NTN
	KC634007	NTN
	KC614702	NTN
Switzerland	MF405303	NTN
	MF422609	NTN
Serbia	KJ946936	NTN
Japan	AB711145	NTN
	AB711143	NTN

Table 14: PVY Isolates/strains used for accessing nucleotide Phylogenetic similarity for "O" Strain

Location	Accession No.	Isolate/Strain
Kashmir	NA	SL1o
	NA	SL3o
	NA	Ant4o
China	HM590405	O/N
	GQ200836	O
	EU182576	O/N
	JQ673517	O/N
	KU375554	O/N:O
Poland	KX356070	O/N-Wi
Brazil	JQ924288	O/N-Wi
	JQ924286	O/N-Wi
	JF928460	O/serotype N
	JF928459	O/serotype N
USA	HQ912896	PVY-Wi
	HQ912868	PVY-Wi
	HQ912863	PVY-Wi
	AY884983	O/N
	AY166867	O/N
UK	HQ912869	O/NTN

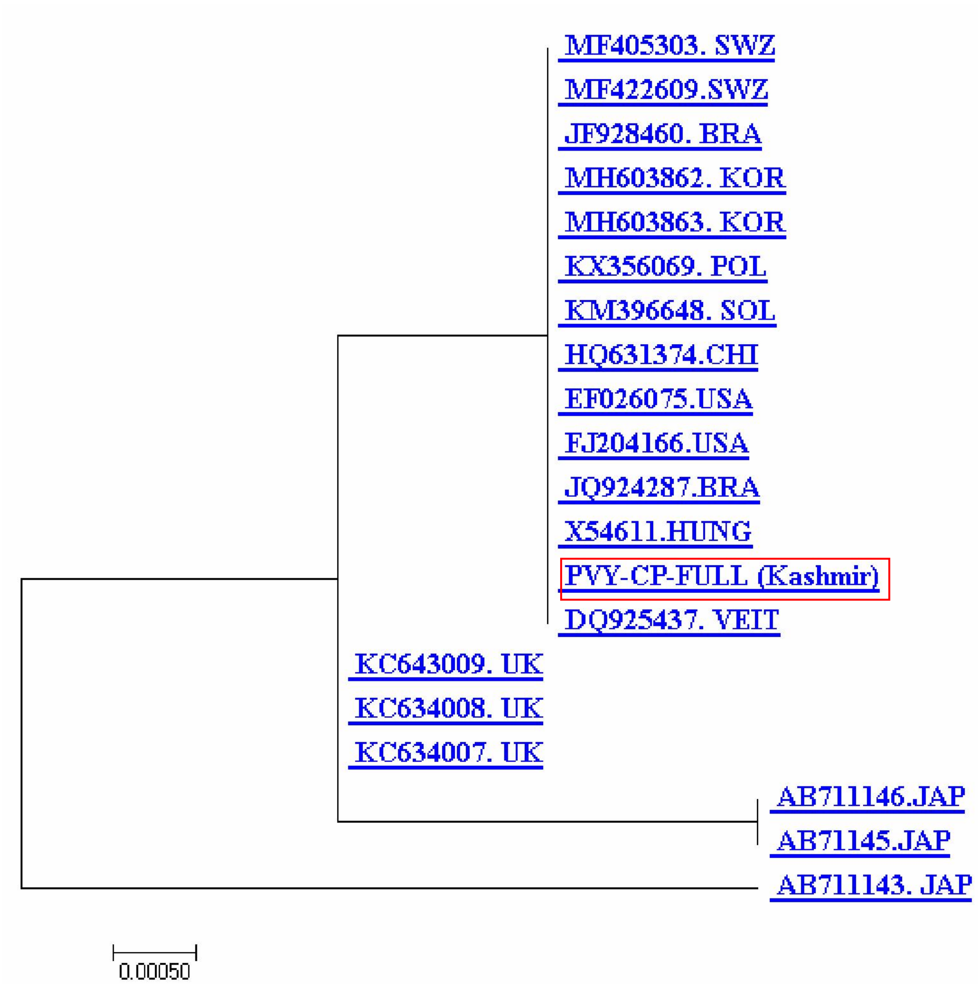


Fig. 2: Phylogenetic analysis of the PVY-CP-Kashmir by Maximum Likelihood method. The tree depicts the relationship of PVY-CP-Kashmir with other PVY isolates at CP level

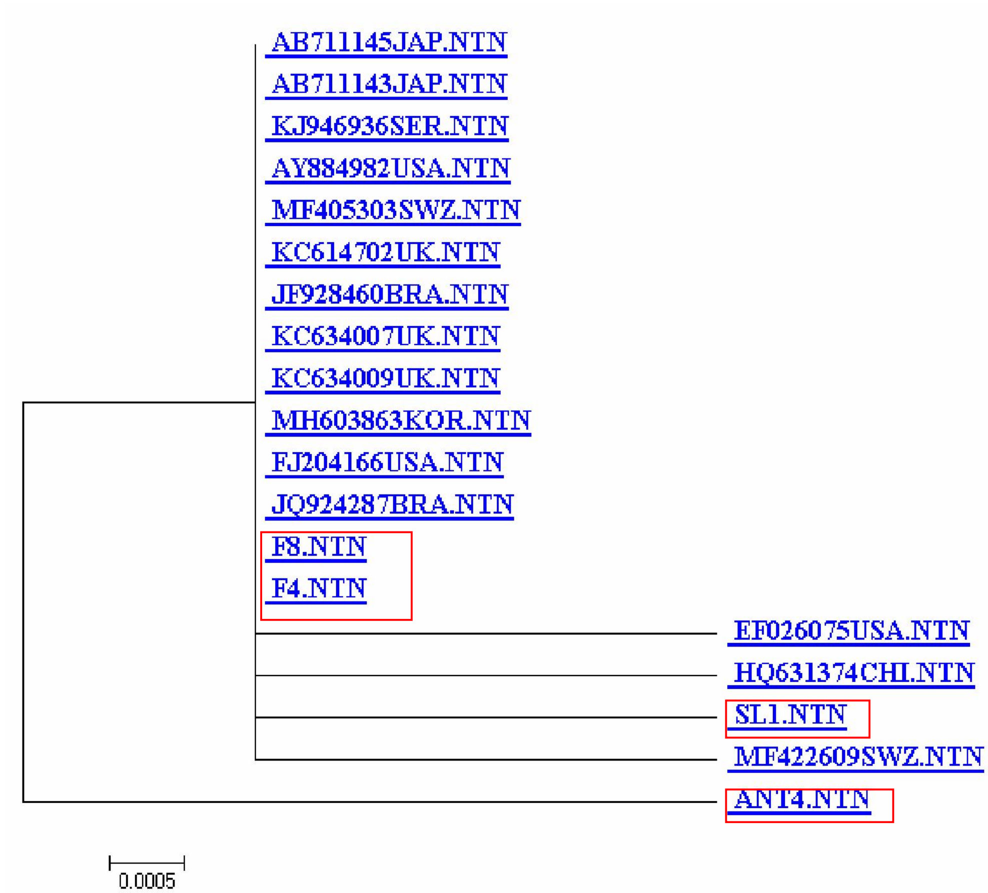


Fig. 3: Phylogenetic analysis of the PVY-NTN Strains by Maximum Likelihood method. The tree depicts the relationship of PVY -NTN-Kashmir Isolates with other PVY - NTN Strains throughout the world

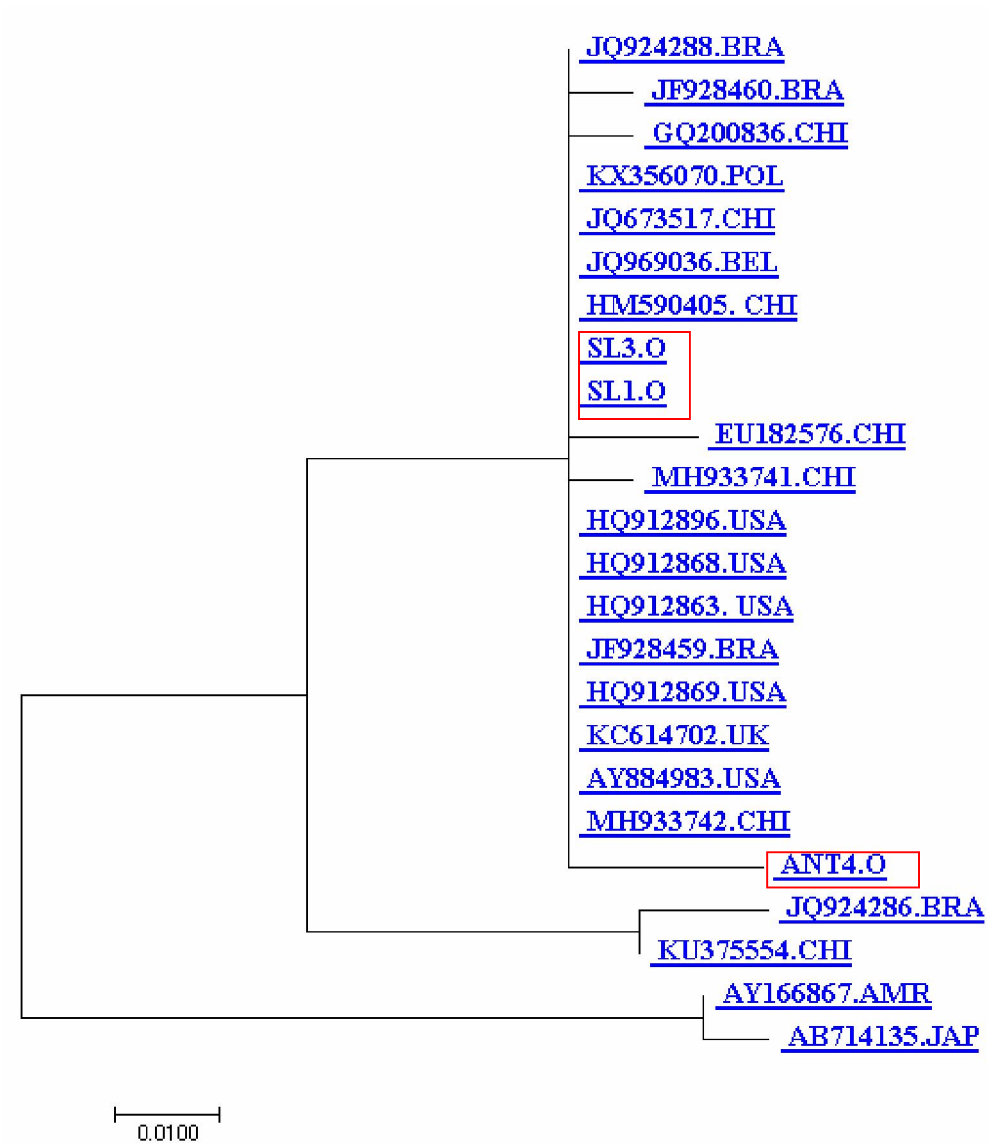


Fig. 4: Phylogenetic analysis of the PVY- "O" Strains by Maximum Likelihood method. The tree depicts the relationship of PVY "O" - Kashmir Isolates with other PVY "O" Strains throughout the world

4.5 Screening of available germplasm

Twenty varieties of potato were grown in the trial pots in greenhouse, SKUAST-K Shalimar. Some potato varieties showed the viral symptoms like mosaic patterns on leaves, malformations, veinal necrosis but some varieties were symptomless. The leaf samples belonging to all the twenty varieties were collected. Using the method of Multiplex PCR the samples were analysed. In addition to PVY, the primers for PVX, PLRV, PVS and PVA were used for this multiplex PCR. Different primers that were used in the screening were gifted to us by Dr. Bikas Mandal, Senior Scientist, Department of virology IARI. Various combinations of mixed infections were determined in the tested samples. The most common mixed infection was PVY+PVX (Table 15).

Table 15: Screening of available germplasm using Multiplex PCR with PVY, PVX, PLRV, PVS and PVA Primers

Pot Number	Variety	Virus				
		PVY	PVX	PLRV	PVS	PVA
P1	Himalayan	+	+	-	-	-
P2	P-17	+	-	-	-	-
P3	Longster Long	+	+	-	-	+
P4	Gulmarg special	+	+	-	-	-
P5	ShalimarPotato-1	-	+	-	-	-
P6	Kufri Badshah	+	+	-	-	+
P7	Kufri Chipsona-1	+	+	-	+	+
P8	Kufri Chipsona-2	-	+	-	-	-
P9	Farmers variety	-	+	-	-	+
P10	Farmers variety	-	+	-	-	-
P11	Farmers variety	+	+	-	+	-
P12	Farmers variety	+	+	-	-	-
P13	Farmers variety	+	+	-	+	-
P14	Farmers variety	+	-	-	-	-
L1	SP-1	-	-	-	-	-
L2	Kufri Giriraj	-	-	-	-	-
L3	P-14	-	-	-	-	-
T1	Tissue culture	+	+	-	-	+
T2	Tissue culture	+		-	-	+
T3	Tissue culture	+	+	-	-	+

Chapter- 5

DISCUSSION

Potato is one of the leading food crops, occupying fourth place in importance after rice, wheat and maize. Potato is known for sustaining millions of lives throughout the world. It is cheap and nutritious food especially in developing countries like India, owing to its high production potential per unit area and time and high nutritional value to sustain burgeoning population (Brown, 2005). In Kashmir valley also, potato is the most important vegetable with wider consumption. Potato crop is prone to systematic infections by viruses, mycoplasmas, fungi, bacteria and nematodes. These biotic stresses effect the plant stand, vigour, foliage, production potential and ultimately the crop yield. The degenerative effect of viral disease is more devastating in potato (as it is vegetatively propagated) and there is no control measure of these diseases at field level. However due to limited information about viruses and the diseases they cause, management of viruses has been overlooked. Furthermore most farmers are not aware of viral diseases and their effect on yield reduction. This is because most viral infections of potatoes are often symptomless, latent or may have confusing symptoms.

Among the most known devastating viruses, Potato Virus Y (PVY) is economically most important virus of potato. One of the reasons for the worldwide distribution of PVY is its high percentage of seed transmission by aphids. Seed growers have the additional concern of having their seed lots downgraded or deemed ineligible for recertification if PVY incidence exceeds the 0.5% certification tolerances. This not only causes shortage of certified seed but can also result in loss of farm income for the seed grower. PVY remains viable in potato tubers and when there is high level of PVY in a seed producer’s field, there is an increased initial inoculum level for next year’s seed potato crop, which leads to increase in the chances of that year’s lots being downgraded or rejected. Another issue is rejection of raw potatoes at potato processing and shipping plants. If strains of PVY that cause tuber symptoms become more common, growers may also face rejections from the companies that process and ship potatoes because of poor quality tubers. Host resistance is believed to be the most efficient means of controlling this virus. However, the inconsistent behaviour of these host varieties over time and space indicates the prevalence of more than one strain of this virus. The development of different reactions/symptoms to virus infection depends on virus strain, potato cultivar and environment. Further, the sequence comparison of the coat proteins of potyviruses have shown that N-terminal of the coat protein varies considerably in length and sequence, and the analysis of genomic sequences has made possible the development of a method for identification and comparison of the viruses strains. Thus the knowledge of variability in the virus is of prime importance in identification, exploitation of resistance sources and their deployment over time and space.

Survey was conducted to record the present status of PVY in Kashmir. Commercial potato growing areas and farmers field were surveyed on the basis of general characteristic symptoms of PVY that include; stunting, leaf mottling, crinkling, yellowing and necrosis. Severe symptoms consist of dark brown, dead areas in stems of nearly mature leaflets. The terminal leaflets sometimes showed severe necrosis, and in many cases, all leaflets were infected. Infected leaflets eventually roll downward giving the plants a dropping appearance. Survey revealed the prevalence of the disease in almost all the potato growing areas of valley with an incidence ranging from 16 to 27.33 per cent. Incidence of PVY in district Anantnag, Baramulla, Budgam and Srinagar was recorded in variable proportions. Maximum being in Shalimar area of district Srinagar (27.33%) and minimum being in the Mazhama area of district Budgam (16%). Variable incidence of the disease in different areas has also been recorded elsewhere by Ghorai *et.al*. (2018) and Attaullah and Arif (2017). Ghorai *et al*. (2018), recorded the incidence of PVY in different localities of Punjab in variable proportion (26.7 – 90 percent). PVY causes disease of global importance and has been reported to cause severe crop losses in India, Pakistan, Europe, Canada and America etc (Attaullah & Muhammad Arif, 2017; Gray *et al*, 2010).

The symptomology is an initial step towards disease diagnosis and is not a reliable method to confirm viral diseases because symptom development is due to different biotic as well as abiotic factors but it can play a vital role in disease diagnosis, as reported by Batool *et al.* (2011). A wide range of techniques are currently available for the detection and identification of plant viruses. These techniques are useful in surveys for virus diseases, disease monitoring in crops, quarantine systems, epidemiological studies and breeding programs to incorporate host plant resistance. The use of a range of different detection methods results in increased sensitivity and specificity and expands the range of applications of the diagnostics in developing effective virus disease management strategies to mitigate the effects of many of the devastating virus diseases (Martin *et al*, 2000). Virus detection based on biological properties and serological assays are by far the most widely used methods but due to the speed, specificity, sensitivity and the power to amplify the target nucleic acid present at an extremely low level, PCR has become an attractive technique for the diagnosis of plant virus diseases (Henson and French 1993; Hadidi *et al*., 1995; Candresse *et al*., 1998).

Therefore the association of PVY with each disease sample in the present study was further established through RT-PCR amplification (as PVY being RNA based virus) of coat protein gene using PVY- specific primers. The PVY-specific primers to amplify coat protein gene of potyvirus/PVY have also been used by different workers (Colinet *et al.*, 1994; Langeveld *et al.*, 1991 and Hamid *et al.*, 2019). The results of RT-PCR also revealed the presence of the PVY in almost all the Potato growing areas of Kashmir with an incidence ranging from 25 to 52.38 per cent, maximum incidence being in the district Srinagar. Samples of Potato virus Y collected from diverse agro-climatic situations of Kashmir were categorized into 31 isolates on the basis of positive RT-PCR results.

Diversity characteristic of positive sense RNA plant viruses is because of three main factors including mutation, recombination and reassortment. All the three factors contribute to development of viral genome pool for new evolutionary niches. PVY, the RNA virus is one such virus that uses mutation and recombination to produce high genetic diversity thus survives and spread in multiple hosts and environment (Beiko *et al*, 2005). In order to breed resistant varieties it is very important to identify and characterize PVY strains. Several PVY strains identified throughout the world are broadly classified under the common and necrotic strain groups respectively (Seo *et al*. 2009). Isolates of PVY largely differ by their pathogenicity properties in differential host species and cultivars (De Bokx and Huttinga. 1981). Based on genome sequences, three major lineages can be distinguished among PVY, named O, C and N (Moury *et al.*, 2002). Only the isolates from the O lineage induce hypersensitive reactions associated to resistance in potato cultivars carrying the *Nytbr* resistance gene, while only the isolates from the C lineage induce similar reactions in potato cultivars carrying the *Nctbr* gene and only the isolates from the N lineage induce systemic veinal necrosis in a set of tobacco cultivars (Kerlan *et al.,* 1999).

Limited information is available regarding the strain composition of PVY populations in various potato-producing states of India. A common strain of PVY (PVY-O) has been identified to be inciting the disease in India based on the sequence analysis of 5'UTR and P1 gene (Rojas *et al*., 1997) and a hybrid strain of PVY-N:O has been identified and characterized to cause infection in potato grown in India (Jailani *et al*, 2017). In Jammu & Kashmir, a northern most state of India, occurrence and complete genome characterization of tuber necrosis strain group (PVY-NTN) in Kashmir has been reported by Hamid *et al*. (2019). However no information regarding status of PVY strains was available in Kashmir.

In the present study 31 isolate were characterized using coat protein specific primers. Further strain identification of these isolates was done using strain specific primers (NTN,N,N;O &O) following (Lorenzen *et al*., 2006). Out of these

31 isolates representing diverse agro- climatic regions of Kashmir valley three strains *viz.,* NTN, N and O were found to be most prevalent with most of the isolates showing mixed infection. The relatively high percentage of mixed infections detected in this study indicates a continued possibility of the emergence of new additional recombinant strains. Potyviruses have been reported to have a relatively high rate of recombination (Revers *et al.,* 1996).

As host resistance is believed to be the most efficient means of controlling this virus, breeding of potato cultivars resistant to PVY infection should take into consideration the full gamut of PVY strains and their relative incidence. Thus, identification of potato genotypes resistant to different strains of PVY infection and incorporation of those genotypes in the potato breeding programs is imperative in order to obtain potato cultivar with broad spectrum resistance. An attempt was therefore made to characterize their coat protein and strain specific genes for comparison. The RT-PCR of the test strains using coat protein specific primer and strain specific primer pair amplified a product of ~ 900 bp and 452bp(NTN), 398bp(N), 267bp (O) respectively. 9 selected isolates (one CP, five "NTN" & three "O") of PVY belonging to three districts of Kashmir (Baramullah, Srinagar and Anantnag) were custom sequenced, sequencing of PCR amplified products revealed that the test sequence consisted of ~900bp, ~452bp, ~ 398bp and ~267bp. The sequences obtained were compared with sequences of PVY obtained from NCBI gene bank http://www.ncbi.gov/blast (Hamid *et al*., 2019). The pair-wise nucleotide homology between various sequences varied between 88 to 100 per cent. The Phylogenetic analysis of CP and strain specific sequences of PVY from Kashmir valley were most closely related to PVY isolates from Brazil, USA, China and other countries. This is the first report of two new strains (ie; O & N) of PVY in Kashmiri potato with nucleotide evidence, as the NTN strain has been already reported by Hamid *et al.* (2019).

Further in this study the available potato germplasm was screened against some economically important viruses of potato like PLRV, PVX, PVS, PVA in addition to PVY. All the potato varieties were found to be infected with one or more viruses. The result of Multiplex PCR showed that the most common viruses in the collected samples were PVY and PVX. These viruses are known to be transmitted mechanically, by contact with diseased plants in nature, through tubers, and by aphids (Hooker, 1986; Burrows and Zitter, 2005). Also in this study PVY “NTN” strain has been isolated from aphids collected from PVY infected plants. The occurrence and wide distribution of PVY and PVX in potato plants were most likely related to the large abundance of aphids in this region. Thus, control of vectors is one of the most important methods for the management of potato viruses in this region. Besides, in consequence of the infestation of all potato varieties in the region with the viruses, use of certified seed (potato tubers) and resistant varieties is necessary for virus-free potato production.

Chapter-6

SUMMARY AND CONCLUSION

The present study entitled "Molecular Characterization of Potato Virus Y (PVY) and its existing status in Kashmir" was carried out to record the incidence of PVY in Kashmir and also to carry out its molecular characterization which involved confirmation of the disease by using coat protein specific primers and then identification of its various strains by using strain specific primers.

The outcome of this study showed the existence of PVY in the different commercial potato growing areas of Kashmir in varied percentages. Some correlations were drawn regarding to the location of potato fields and to the time of planting indicating the success in the virus transmission by aphids. To our knowledge this is the first study of symptomology and molecular analysis of PVY that infect potatoes in Kashmir. Furthermore this study was able to report genetic variation among the PVY by using molecular tool i.e. RT-PCR. Available varieties of potato were also screened against different economically important viral diseases and all the potato varieties were found to be infected with one or more viruses.

Even though this study is highly recommended for further research to cover the variability among the PVY strains that possibly exists in Kashmir. And finally this study re-emphasized the reliability, simplicity and specificity of using RT-PCR for diagnosis and investigation of viral diseases.

CONCLUSION

1) The present study gave us the brief insight about the prevalence and spread of PVY infection in commercial potato fields of Kashmir valley.

2) The availability of specific primers will facilitate the typing of PVY infection and various isolates to the strain level.

3) The study laid the ground for further investigation to determine the extent of PVY infection in particular and possibility of other strains and other viruses in seed and commercial potato fields

4) Breeding of potato cultivars resistant to PVY infection should take into consideration the full gamut of PVY strains.

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CERTIFICATE

Certified that all the corrections/amendments as suggested by External Examiner Dr. S. K. Singh, Sr. Scientist (Plant Pathology) OFRC, SKUAST-Jammu during Viva-Voce examination held on 02-12-2019 have been incorporated in the manuscript entitled **"Molecular Characterization of Potato Virus Y (PVY) and its Existing Status in Kashmir"** submitted by **Ms. Sumiah Wani (Regd. No. 2017-H-176-M).**

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