Incidence of *Potato Virus Y* (*PVY*) in Selected Districts of Bangladesh and It's Molecular Detection

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JUNE, 2018

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REG. NO. 12-04872

A Thesis

Submitted to the Faculty of Agriculture,
Sher-e-Bangla Agricultural University, Dhaka,
In partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

IN

PLANT PATHOLOGY

SEMESTER: JANUARY- JUNE, 2018

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This is to certify that the thesis entitled **Incidence of *Potato Virus Y* (*PVY*) in Selected Districts of Bangladesh and It's Molecular Detection** submitted to the Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of Master of Science in Plant Pathology, embodies the results of a piece of bonafide research work carried out by **Registration No. 12-04872** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

Dated: 20/10/2019
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Dedicated to
My Beloved Parents
&
The Farmers who feed the nation

ACKNOWLEDGEMENT

All praises are due to Almighty Allah for best owing mercy upon Author and for imbibing confidence on author to materialize the research work. It is a great gratification to articulate author's gratitude author's respected parents, who entitled much hardship inspiring for prosecuting author's studies, thereby receiving proper education.

The author feels proud to express her cordial gratitude, deep sense of respect and enormous indebtedness to author's research supervisor Dr.Md. Belal Hossain, Professor, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, for his scholastic supervision, incessant encouragement, positive suggestion and unvarying inspiration all through the research work and for taking massive care in preparing this manuscript. His insight and professional skill have made a distinctive contribution to complete this piece of research.

The author expresses her sincere appreciation, sense of gratitude, respect and immense indebtedness to the respected Co-supervisor Dr.M. Salahuddin M. Chowdhury Professor, Department of Plant Pathology, SAU. for his constant guidance,timely directions and inspirations throughout the tenure of research work.

The author is greatly thankful to her respectable teacher Dr. Khadija Akhter, Professor and Chairman, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, for her valuable teaching, encouragement and co-operation during the entire study period.

The author would like to express cordial thanks to her friends, well wishers Ratna Akter, Mst Aysha Akter, Md Shariful Islam and Md Kawser who helped the author with their valuable suggestions and directions during the preparation of this thesis paper.

The author takes an opportunity to express her cordial thanks and sincere gratitude to the staff of the Department of Plant Pathology, SAU for their valuable co-operation and help to complete this piece of research.

The author expresses her massive thankfulness to all of them who supported and encouraged her to achieve advanced education and regret for her inability for not to mention every one by name.

The Author

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ABSTRACT

Incidence of *potato virus Y* in the three selected potato growing areas of Bangladesh, viz. Savar, Manikgonj and Munshiganj were investigated from field samples. Overall, three upazilla (sub-station) from each district (which were at least 20 km apart) were selected for samples collection and disease incidence monitoring. Collected samples were storage at -20°C and analyzed in Molecular Biology and Plant Virology lab, Central Research Laboratory of Sher-e-Bangla Agricultural University (SAU), Dhaka. In the all investigated areas, *PVY* appeared in severe to moderate level and their incidence was 3-17%. The sources of potato seed tubers were studied and observed that in most of the cases farmers are continuously cultivating same field and used their own seed tubers that was kept in cold storage condition. The frequency of the storage at cold storage was 67.6% which was the highest among the all sources. In this study, *PVY* was detected through reverse transcriptase polymerase chain reaction (RT-PCR). A 480 bp amplicon of *PVY*- coat protein (CP) gene was amplified and the nucleotide sequences was also amplified in this study.

LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

List of Figures (Contd.)

LIST OF APPENDICES

List of Abbreviations

bp	Base pair
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide Triphosphate
dsRNA	Double stranded RNA
EDTA	Ethylinediamine Tetra acetic acid
GUS	β-glucuronidase
Kb	Kilobase
mRNA	Messenger RNA
O.D	Optical Density
PCR	Polymerase Chain Reaction
PVY	*Potato virus Y*
PLRV	Potato Leaf Roll Virus
PVY-CP	*PVY*-Coat Protein
PTGS	Post-transcriptional Gene Silencing
RISC	RNA-Induced Silencing Complex
RNAi	RNA interference
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
siRNA	Small Interfering RNA

INTRODUCTION

Potato belongs to genus *Solanum* is a large genus consisting of about 1000 species. More than 200 species of potato have been found, but just eight are cultivated for the last 2000 years (Smith, 1977). Among the most important food crops in the world, Potato (*Solanum tuberosum* L.) is in the fourth position next to wheat, rice and maize (Islam, *et al*., 2014). The potato tuber is an excellent source of carbohydrates, protein and vitamins (MacGillivary, 1953). In the world, potato is cultivated 19,098,300.00 ha of lands and production 381,682,000.00 tons (FAOSTAT, *et al*., 2017) and in Bangladesh, cultivated 4,75,488 ha of lands and production 94,74,098 metric tons (BBS 2016-17). Potato is cultivated twenty three major growing areas in Bangladesh. In comparison to other agricultural crops, the seed cost of potato cultivation is much higher. Bangladesh Agricultural Development Corporation (BADC) reported that the seed cost of potato is liable to 30-40% of total production cost (Anon, 2005). Bangladesh imported high yielding foreign potato varieties at the cost of above US $ 150.00 per quintal which engross large amount of foreign currency every year (Anon, 2010). High yielding foreign potato varieties significantly increased the yield of potato crop in our country but at the same time resulted new viral problems like *PLRV* (*potato leaf roll virus*), *PVY* (*potato leaf roll virus*) and *PVX* (*potato virus X*)which have been reported in Bangladesh and causes 10-90% yield losses. Quality seed production in the country is mainly threatened by viruses like *potato virus Y (PVY), potato virus X (PVX), potato virus M (PVM), potato virus A (PVA), potato leaf roll virus (PLRV)* and virus like substances like *potato spindle tuber viroid (PSTV).* Infection of any one alone or some of them jointly would retard plant growth and reduce tuber yield. The infected tubers cannot be used for seed. Despite fungal and bacterial diseases, post-emerging control of viruses and viroid is not applicable. Potato seed tuber quality includes two aspects- a) the level of disease infection and b) the physiological age of seed tubers. Seed tubers which are planted continuously for

several years, show degeneration. This degeneration is aroused by several kinds of viruses and virus-like organisms. Because of asexual propagation, viruses and viroids can be accumulated in tubers, which ultimately lead to degeneration of the potato. The worldwide production of potato is seriously affected by *potato virus Y (PVY)* (Wolf and Horvath, 2000). *PVY* is mainly transmitted through mechanical means and by aphid species in a non-persistent manner (Brunt, 2001). Alone or combine effect of *PVY* strains can cause yield losses up to 10 to 90% and decrease plant productivity and tuber quality (Novy et al., 2002; Brunt, 2001). According to its genetic structure, *PVY* belongs to the genus Potyvirus with 9.4 kb genome consisting of a single strand RNA molecule with plus orientation that contains a single extended open reading frame and directly acts as mRNA. It occurs as long, flexuous particle measuring 730x11nm. Disease symptoms prevailed by *PVY* vary from an almost slight mosaic to severe necrosis and early death of plants. The severity of infection depends on cultivar and viral strain (Souza Dias and Imauti, 1997). The goal of this study is to detect *PVY* through RT-PCR amplification and sequencing of *CP* gene for characterization of *PVY* Bangladeshi isolate. Through this study, a reliable RT-PCR based molecular detection method will be developed for local strain of *PVY*, and a Bangladeshi isolate of *PVY* would be found on the basis of nucleotide sequencing from cloned *CP* gene. The sequence identity analysis of the cloned *PVY -CP* gene will be used to assess homologies among several *PVY* isolates reported in Genbank database.

Objectives

The specific objectives of the proposed study are as follows-

- To investigate the disease incidence level of *PVY* in selected area of Bangladesh.
- To identify local strain of *PVY* by using a reliable RT-PCR based molecular detection method.
- To explore and assess homologies among several *PVY* isolates reported in GenBank database.

REVIEW OF LITERATURE

Potato (*Solanum tuberosum* L.) is an important crop and one of the leading vegetables in the world including Bangladesh. Potato is a tasty, nutritive and highly digestible vegetable with 75 per cent water contents. One hundred grams of potato possesses 22g carbohydrate, two gram protein, 90 kilocalories energy, 13mg calcium, 17mg vitamin C, 11mg riboflavin 1.2 mg niacin and traces of certain other minerals such as calcium, iron, magnesium, phosphorus, potassium, sodium, and sulfur and fibre. Potato has medical significance. It is free of cholesterol and also contains some antioxidants which are capable of protecting human beings against cancer and heart diseases. It has potential to lower high blood pressures due to presence of a compound kukoamine. Potato tubers if exposed to sunlight during growth become green in color due to formation of poisonous alkaloid compounds solanine and chaconin which are injurious for humans as well as animals and can even cause death.

Table 1. Nutritive value of potato (per 100 gm edible portion)

Nutrients	Content
Edible portion	85%
Moisture	74.70 gm
Protein	1.60 gm
Fat	0.10 gm
Minerals	0.60 gm
Carbohydrates	22.60 gm
Energy	97.00 kcal
Calcium	10.00 mg
Phosphorus	40.00 mg
Iron	0.70 mg
Carotene	24.00 mg
Thiamine	0.10 mg
Riboflavin	0.01 mg
Niacin	1.20 mg
Vitamin C	17.0 mg

2.1. Potato Viruses

Potato is propagated by taking the vegetative part of the plant, called the tuber and planting it in soil. Pathogens that establish a systemic infection persist in the vegetative parts of the plant and are transmitted generation after generation through the vegetative propagative cycle. Therefore, it is necessary to prevent the infection by these pathogens. The most important pathogens are the viruses which include major viruses namely Potato virus *Y* (*PVY*), Potato virus *X* (*PVX*), Potato leaf roll virus (*PLRV*) and minor viruses including Potato virus *M* (*PVM*), Potato virus *S* (*PVS*) and Potato virus *A* (*PVA*), Potato spindle tuber viroid *(PSTV). PVY* belongs to the family *Potyviridae* and its genome is composed of a single-stranded, positive-sense RNA molecule of about 9.4 kb. During the infection process, this RNA is translated into a large precursor polyprotein that is cleaved co- and post-translationally into 10 mature proteins (Dougherty and Carrington, 1988).

2.2. Molecular Description of *PVY*

Potyvirus virions consist of non-enveloped filamentous structures that are 680 – 900 nm in length and 11 to 15 nm in width (Edwardson, 1947). Morphologically the potyvirus consists of approximately 2,000 copies of coat protein (CP) which forms a cylindrical inclusion body (CIb) (Talbo, 2004). The CIb is considered to be the single most important phenotypic criterion for distinguishing a potyvirus from other virus groups.

The CIb encapsulates a single strand of positive sense RNA which is in the order of 10 kb in length and has a non-translated 5‟-terminal region (5‟-NTR) as well as a 3‟-poly-A tail (Dougherty and Carrington, 1988). The 144 nucleotide 5‟-NTR is particularly rich in adenine residues and has very few guanine residues. Rather than a conventional cap structure, the 5‟NTR is associated with a viral genome linked protein (VPg) which is said to act as an enhancer of transcription (Carrington and Freed, 1990).

The 5‟-leader sequence has an internal ribosome entry site (IRES) and cap-independent translation regulatory elements (CIREs) (Dallaire *et al*., 1994). The IRES directs cap independent translation through a mechanism similar to that used by eukaryotes (Niepel and Gallie, 1999). The extended open reading frame encodes for a 350 kDa polyprotein. This polyprotein is proteolytically processed by viral proteases (NIa, HC-Pro and P1) and undergoes co- and posttranslational cleavage to yield several multi-functional proteins. These include the following-P1 (P1 Protein), HC-Pro (Helper Component Proteinase), P3 (P3 Protein), 6K1 (6-kDa Protein 1), CIb (Cylindrical Inclusion body), 6K2 (6-kDa Protein 2), VPg (Viral Genome-linked Protein), NIa-Pro (Nuclear Inclusion Protein a, Proteinase domain), NIb (Nuclear Inclusion Protein b) and the CP (Coat Protein) (Talbot, 2004).

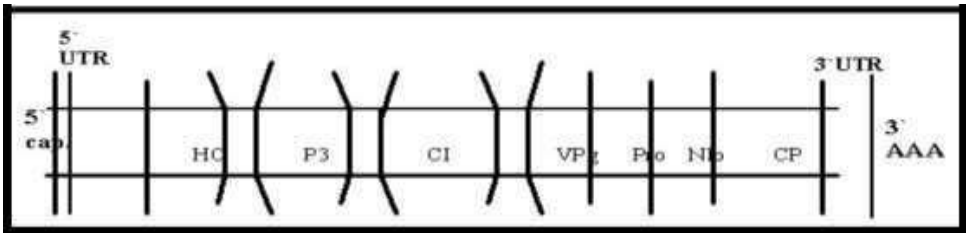

Figure 1.Genome map of Potato virus*Y*. Leiser & Richter, (1978) stated that virions contain 5.4-6.4% nucleic acid and one molecule of linear single stranded RNA total genome length

2.3. *PVY* Biology

PVY is one of the most economically important pathogen of potato largely widespread in potato and is responsible for significant reduction of yield (up to 80%) and tuber quality. Apart from potato it infects pepper, tomato, tobacco and many other plants. *PVY* is transmitted with aphids (*Myzus persicae*) most important), mechanically and through infected tubers. Within minutes of starting to feed on a *PVY*-infected plant, the *PVY* particles get stuck in the aphid stylet

(its piercing-sucking mouthpart). If the aphid then moves to a healthy plant and soon starts to feed, the virus particles are transmitted to the healthy plant. This process is termed as “non-persistent” transmission.

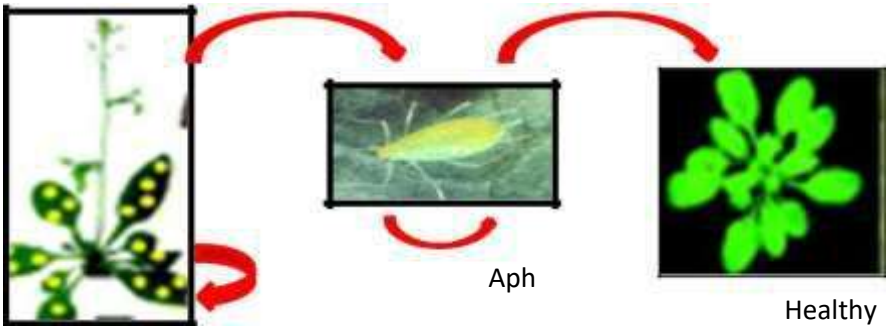

Figure 2. *PVY* transmission from infected to healthy plant

2.4. *PVY* Replication inside plant

Upon entrance into the plant cell, the virus coat protein disassembles and releases its RNA genome. The viral RNA serves as mRNA, and although little is known about the translation thereof, it is believed that the 5‟ non-coding region functions as an enhancer of translation (Carrington and Freed, 1990). The translated mRNA results in a polyprotein which is processed into mature proteins. Each polyprotein is then cleaved into ten different proteins which are believed to be multifunctional. These proteins, along with host proteins, assemble to form a replication complex. This complex performs negative-strand RNA synthesis, using the positive strand of viral RNA as a template. Once the additional RNA copies have been produced, they code for the synthesis of various proteins as well as coat proteins. These coat proteins will now enclose the newly formed genomes to give rise to new virions. It has been suggested that enclosure of the newly formed virions is initiated by the interaction of the coat proteins with the 5‟terminus and that the coat protein is built up towards the 3‟terminus (Wu and Shaw, 1998). The entire process of viral replication occurs within the endoplasmic reticulum. These newly synthesized

viral particles are subsequently transported through the plasmodesmata to adjacent plant cells via several assisting *potyvirus* proteins. Distribution of viruses within the plant occurs according to the source-sink relationship between maturing and growing tissues (Talbot, 2004). Virus concentration throughout the plant is high and this greatly increases the chance of uptake by aphids.

2.5. Symptoms of *PVY*

According to symptoms in test plants, the *PVY* isolates infecting potato were historically classified into different strains- PVY^{N} (tobacco veinal necrosis), PVY^{O} common), PVY^{C} (potato stipple streak) and PVY^{NTN}, distinct group inside the PVY^{N}. Symptoms of *PVY* infection in potato include yellow / light green / dark green mosaic patterns on leaves, leaf distortion, brown / black (necrotic) line patterns often on veins or shoots, stunted growth, death of growing points, and tuber necrosis. Symptoms can vary from severe in highly susceptible cultivars to mild or absent in highly tolerant or resistant cultivars. Symptoms are plant genotype, virus isolate and temperature dependent (Souza Dias and Iamauti, 1997).

Figure 3. Symptoms of *potato virus Y*, mosaic and necrotic leaves can be clearly seen

2.6. Plant-Virus Interactions

Viruses are small, obligate parasites consisting of genomic material packed into particles that are entirely dependent on their host to sustain their life cycle. Successful establishment of viral infection alters plant metabolism significantly since viruses divert valuable resources such as amino acids, nucleic acids and proteins required for basic metabolism in plants to viral replication.

Viruses that infect plants are generally single-stranded(ss) positive-sense RNA viruses. The accumulation of the virus progeny inside the plant cells involves translation, replication, cell–to-cell and long-distance movement of viral sequences. Viruses can only enter the plant cell passively through wounds caused by physical injuries due to environmental factors or by vectors. Among vectors, several species of insects, mites, nematodes and some soil inhabitant fungi can transmit specific viruses. In the cytoplasm, the RNA disassembles replicates, converts its mRNA to proteins, and mobilizes locally and systemically. Viruses use energy and proteins from the host cell to perform these processes (Stange, 2006).

Different interactions are generated between the plant and the virus during each stage of the viral cycle. If the viral particle is not recognized by the host plant, a compatible interaction between the plant and the virus is established. This interaction may be favorable for the virus (Hammond-Kosack and Jones, 2000). However, if the plant recognizes the viral particle, an incompatible interaction that is unfavorable for the virus is established. It is known that plants can recognize the virus, limiting it to the site of the infection. A series of complex cascade defence reactions can be induced, limiting virus replication and virus movement within the host plant (Hammond-Kosack and Jones, 2000).

For DNA and RNA type plant viruses, viral accumulation within plant cells depends on replication and translation processes (Ahlquist et al., 2003).

Cascade followed by a virus when enter inside a plant involves following steps-

1) Disassembly of virus particle
2) Replication and translation using host cell machinery
3) Assembly and movement of progeny viruses
    i) Cell-cell movement
    ii) Long distance movement

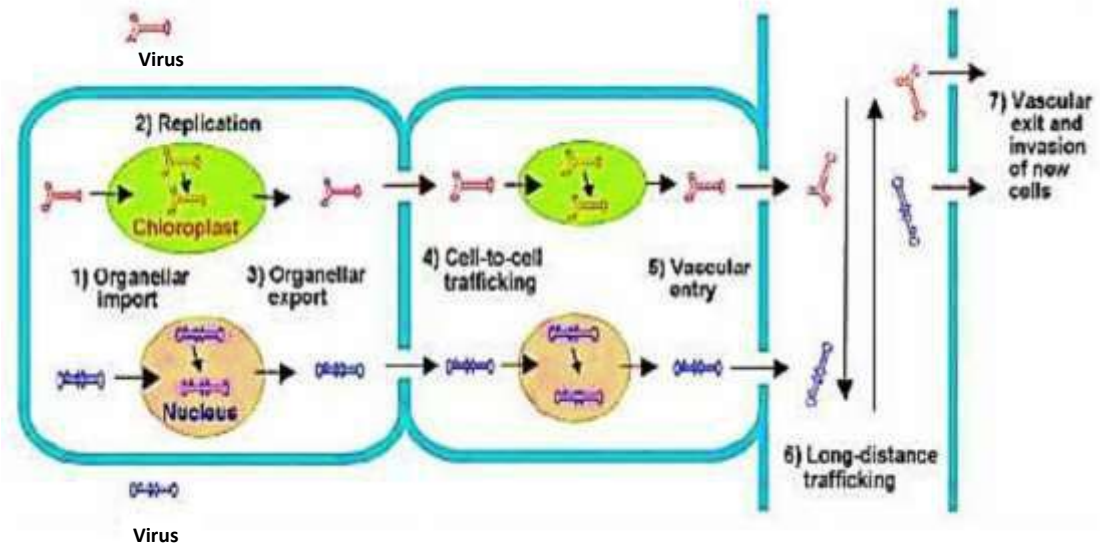

Figure 4.Entry of viral particle in plant cell and its cell to cell movement and to other plant parts.

2.7. Strategies to overcome viral invasion

2.7.1. Hypersensitive Response

Pathogens have developed counter measures that are able to suppress basal resistance in certain plant species. If a pathogen is capable of suppressing basal defense, plants may respond with another line of defense-the hypersensitive response (HR). The HR is characterized by deliberate plant cell suicide at the site of infection. Although drastic compared to basal resistance, the HR may limit

pathogen access to water and nutrients by sacrificing a few cells in order to save the rest of the plant. The HR is typically more pathogen-specific than basal resistance and is often triggered when gene products in the plant cell recognize the presence of specific disease-causing effector molecules introduced into the host by the pathogen. Bacteria, fungi, viruses, and microscopic worms called nematodes are capable of inducing the HR in plants (Brian *et al*., 2008).

2.7.2. Pathogen gene mediated resistance

Cross protection has been applied to generate viral gene transgenic plants to control virus infection. Various viral or sub viral genes have been transformed into plant genome to generate virus resistant plants. According to the gene used in plant transformation different names were given to describe different resistance mechanism involved, such as coat protein mediated resistance (Beachy *et al*., 1990), Satellite RNA mediated resistance (Baulcombe *et al*., 1986), replicase gene mediated resistance (Anderson *et al*.,1992). Cross-protection is thought to be a complex response caused by the replication and expression of the entire viral genome.

2.7.3. RNA mediated Resistance (RNA Silencing)

Virus resistance is achieved usually through the antiviral pathways of RNA silencing, a natural defense mechanism of plants against viruses. The experimental approach consists of isolating a segment of the viral genome itself and transferring it into the genome of a susceptible plant. Integrating a viral gene fragment into a host genome does not cause disease (the entire viral genome is needed to cause disease). Instead, the plants natural antiviral mechanism that acts against a virus by degrading its genetic material in a nucleotide sequence specific manner via a cascade of events involving numerous proteins, including ribonucleases (enzymes that cleave RNA) is activated. This targeted degradation of the genome of an invader virus protects plants from virus infection. In plants, RNAi is a major

Molecular defense mechanism against viruses (Ding and Ovine, 2007; Ovine, 2005). Although primarily initiated by dsRNA in the cytoplasm, RNAi is also activated in viroid-infected plants. Double-stranded replicative intermediates of viroids are generated in the nucleus or in chloroplasts (Tabler and Tsagris, 2004) where they can hardly trigger RNAi.

2.7.4. Protein mediated Resistance

Viral derived transgene can confer both protein- and RNA-mediated protection. The attribution of resistance to expression of the viral protein or to its RNA is often posed as a „dilemma". Several explanations have been proposed to reconcile different and sometimes contrasting results. However, in spite of uncertainty about mechanisms, high levels or broad resistance may be attributed to co-existence of both protein- and RNA-mediated interferences (Prins *et al*., 2008).

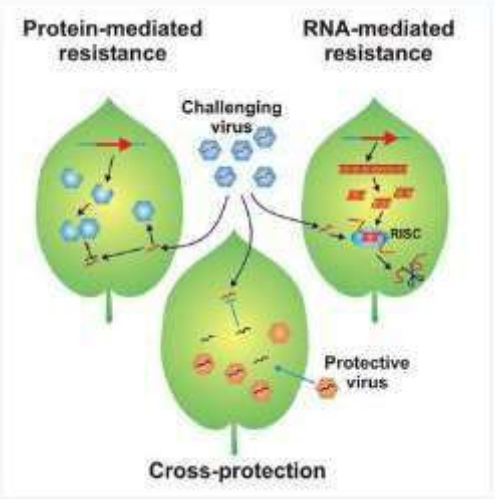

Figure 5. Basic strategies used in plants to confer resistance against viral attack

2.8. RNA Silencing; Innate Immunity against Viruses in Plants

Viral diseases are a major threat to world agriculture and breeding resistant varieties against these viruses is one of the major challenge faced by plant virologists and biotechnologists. The development of the concept of pathogen

derived resistance gave rise to strategies ranging from coat protein based interference of virus propagation to RNA mediated virus gene silencing. Much progress has been achieved in protecting plants against these RNA and DNA viruses. In plants, silencing is of two types-transcriptional and post transcriptional gene silencing. Both types operate at RNA level.

2.9. RNA-Mediated Transcriptional Silencing

RNA silencing in plants is often associated with changes to the methylation of histones and DNA at the target gene locus (Mathieu and Bender, 2004). The basic mechanism of RNA silencing does not explain a link with chromatin/DNA modification. However, it has been known since 1994 that there is the potential for such a link because DNA methylation in plants can be directed by RNA (Wassenegger *et al*., 1994).

2.10. Protection against viral infections

RNAi can work as a defense mechanism against viral attacks. It has been shown earlier that plant cells have an efficient defense against viruses based on the PTGS phenomenon (Covey *et al*., 1997; Ratcliff *et al*., 1997). Today, we know that RNAi based anti-viral mechanism is at work in plants, worms and flies, whereas it is still uncertain as to how relevant it is for vertebrates, including man.

MATERIALS AND METHODS

Diseases play an important role in reducing the quality and quantity of cultivated crops. The present study was conducted to study the incidence level of *potato virus Y* in the selected growing areas of Bangladesh and their molecular detection through RT-PCR. This chapter deals with two experiments and the experiments were as follows-

3.1. Experiment-I: Collection of *PVY* +ve samples from the selected potato growing areas of Bangladesh

3.1.1. Survey of selected potato growing areas

During the year 2018-19, a survey was conducted in the three selected potato growing areas of Bangladesh viz Savar, Manikganj and Munshiganj. Overall, three upazilla (sub-station) from each district (which were at least 20 km apart) were selected for samples collection and disease incidence were monitoring. Basic information regarding potato crop viz. varieties, sources of seed tubers, irrigation, agricultural practices and disease incidence etc. was also collected from farmers/growers of all areas through a structured questionnaire.

3.1.2. Sampling

Virus infected leaves/tubers samples were collected. At least 10 random and 10 non-random samples were collected from each upazilla (sub-station) in farmers field by moving diagonally in the field. A total of 180 samples of 6-8 weeks old field growing potato plants were collected on the basis of virus and viral like symptoms. A single sample was consisted of three single leaflets taken from top, middle and bottom, and placed in polythene sample bag and stored in an ice box. Samples were labeled appropriately to indicate location, sample number and date of collection. These samples were brought to Molecular Biology and Plant

Virology lab. under the Department of Plant Pathology Sher-e-Bangla Agricultural University (SAU), Dhaka-1207. All samples were stored at -20°C.

3.1.3. Observation of the Symptoms

Symptoms of the diseases were studied by visual observation. Sometimes hand lens was used for critical observation of the disease and sometimes a disease was identified based on matching the observed symptoms in the infected leaves and tuber with the symptoms.

3.1.4. Collection of Diseased Specimen and Processing

Potato tubers were collected from infected disease plant. The specimens were preserved in the laboratory room following standard procedure of preservation. The disease specimens were arranged in district wise and preserved until the RNA extraction was made.

3.2. Determination of Disease Incidence

Data were collected from farmer's field during potato cultivation to observe disease incidence in potato. Randomly and Non-random by 544 m^2 (435m × 5m)/4 area was selected in farmers field. Total leaves and tuber were counted from 544 m^2 selected area and among them disease infested leaves and tubers were counted to calculate percent disease incidence. All data were collected from 3 districts with selected locations followed by a prepared simple questionnaire.

$$\text{Disease incidence (\%)} = \frac{\text{Total Number of Infected Plants}}{\text{Total number of Investigated Plants}} \times 100$$

3.3. Data Analysis

The collected data was arranged then put in the excel sheet and analysis by using the computer basis software SPSS.

3.4. Experiment-II: Molecular detection of *PVY* through RT-PCR Test

3.4.1. Primer Designing

Primers were designed by using primer-3 latest version software. During the primers selection multiple alignments were done by using Clustal-W latest version software for identification the conserved regions. The following primers pair was used in amplification of *PVY* CP-gene.

Table 2. Primer Sequences used in amplification of *PVY*-CP-gene.

Primer ID	Sequence (5'-3')	PCR product size(bp)
PVY480-FP	ACGTCCAAAATGAGAATGC	**480**
PVY480-RP	TGGTGTTCGTGATGTGACCT	

3.4.2. Extraction of RNA from *PVY* +ve leaves samples

Total RNA was extracted from *PVY*+ve experimental host plant leaves that maintained in the net house, by the "single step" method described by Chrzanowska.

Leaves were collected in liquid N_2 and ground to fine powder with already cooled pestle and mortar

Ground samples were shifted to 1.5 ml tubes

TRIzol @ one ml per 0.2g of ground samples was added and kept for 5 minutes at room temperature for dissociation of nucleoprotein complex, completely.

Chloroform 0.2 ml per 01 ml of TRIzol was poured before through shaking for 30-45 seconds.

Then, it was put at room temperature for 5-10 minutes.

After this incubation, centrifugation on 13000rpm for at 4°C 15 minutes was given.

The RNA rich upper portion of the centrifuged solution was poured into another Eppendorf.

The precipitation of RNA was achieved by pouring 0.5 ml isopropanol.

Ten minutes of incubation time was given to samples followed by centrifugation for 10-12 minutes with 13000 rpm at 4°C.

Washing of pellet was done by adding 01ml ethanol (75%) and supernatant was removed.

The sample was re-suspended with ethanol (75%) properly through pipetting and centrifuged at 10000 speeds for 5-6 minutes and temperature was 4°C.

Then remove supernatant and air dry for 10 minutes time of RNA pellet.

DEPC treated water (20µl) was used to resuspend RNA and put at -20°C. After quantification through spectrophotometer RNA quality was confirmed1% agarose gel

Sampling

Grinding

Adding Trizol

Adding Chloroform

Centrifugation

Adding Isopropanol

Incubate for 10 minutes

Drying pellets

Resuspending RNA with DEPC treated water

Figure 6. Steps of RNA Extraction

3.4.3. Complementary DNA (cDNA) Synthesis

"First Strand cDNA Synthesis kit" (Thermo) was used to synthesize cDNA. The cDNA was synthesized by adding the total RNA @ 1µg plus 1.0µl of the reverse primer i.e.10 pmoles plus nuclease-free water to make final volume at 11µl and mixed slightly and spun gently in a micro-centrifuge for 3-5 seconds. After, giving 5 minutes of incubation at 70^{o}C plus ice chilling plus addition 5X reaction buffer 4µl plus Ribonuclease-inhibitor 1µl (20u/µl) and 2µl of 10mM dNTPs mix 02µl (20u per µl) then incubation at 37^{o}C, 5 minutes and reverse transcriptase 2µl (M-MuLV 20u/µl) for volume of 20µl reaction. Lastly, incubation at 37^{o}C plus 60 minutes incubation time before stopping the reaction and ice chilling at once.

The thermo cycling condition was as follows-

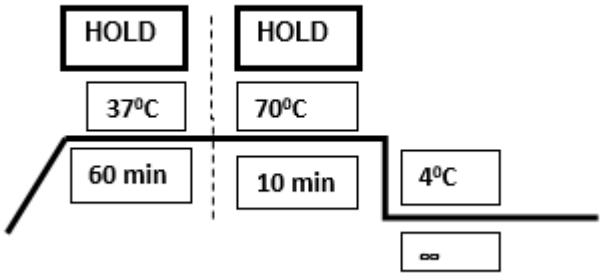

Figure 7. Cycling profile for complementary DNA (cDNA) synthesis

3.4.4. RT–PCR amplification

The 480 bp product was visualized from cDNA of *PVY* by using pair of primers Reverse, 5‟- TGGTGTTCGTGATGTGACCT -3‟ and Forward, 5‟- ACGTCCAAAATGAGAATGC -3‟. PCR conditions are elaborated as shown in the Figure 8.

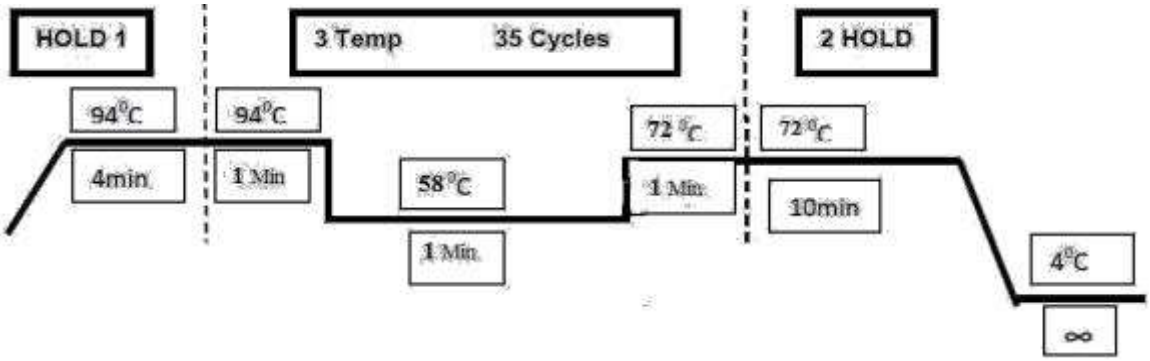

Figure 8. RT-PCR Cycling Conditions to Amplify *PVY-CP* Gene Fragment

3.4.5. Agarose gel electrophoresis

Total 1.5% of Agarose gel mixed with TBE buffer 1x helped to resolve the RT-PCR products . To confirm the product size of the RT-PCR, 50 bp DNA ladder (Thermo) was used as marker. Before visualizing 480 bp product under UV light, gel was stained with ethidium bromide Gel Doc-2000.

3.4.6. PCR product purification

The 480 bp DNA fragments(PCR product) were cut precisely around the boundary of band by using a sterile scalpel blade. Precise cut increases the chances of better elution in the subsequent step. Placed the cut portion of gel slices in a weighed Eppendorf tube of 1.5ml size and again weighed. Gel slice weigh was recorded as this was going to be used as clue for binding buffer. The gel slice was added with binding buffer at 3:1. A temperature of 58°C was adjusted for 5-10 minutes incubation of gel mixtures, to help the dissolving process this mixture was inverted rapidly for 5-10 times, gently. The re-suspended 5 µl of Silica Powder suspension was mixed with the DNA/binding buffer. At 58°C, this mixture was incubated for 5 minutes to allow DNA adhesion with matrix of the silica. To keep suspension of the silica powder, vortexing was done. Pellet from DNA mixture/silica powder was separated after 5 seconds of centrifugation. Washing buffer 500µl ice cooled and diluted ethanol was added as described by the manufacturer, the pellet was remixed and spun for 5 seconds. Then step was repeated for 3 times after discarding the supernatant. After the last wash, supernatant was removed and again the tube was centrifuged to separate all the liquid contents. 5-10 minutes time was given to pellets for air drying to get rid of residual ethanol in the purified DNA solution. Then Incubated the tube containing re-suspended pellet in 10 µl of sterile de-ionized water at 58°C for 5 minutes.

3.4.7. Sequencing PCR

Forward and reverse primers were used to amplify the *PVY-CP* gene for sequencing and the sequencing reaction set-up was as follows-

Components	**Quantity**
PCR product	2.0µl
primer (Forward/reverse)	1.5µl
5X sequencing buffer	1.5µl
Big dye	1.0µl
PCR H_2O to be made	10µl

ii) Sequencing thermo cycling conditions

The cycling profile for the sequencing of *PVY- CP* gene is shown as figure 9.

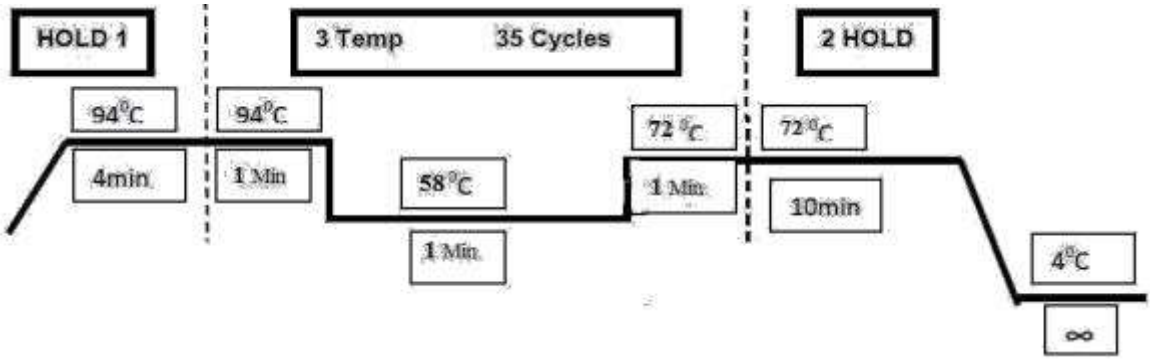

Figure 9. Thermo cycling conditions for sequencing PCR

iii) Purifying sequencing extension products by isopropanol precipitation

PCR product was transferred in label Eppendorf and added 200µl of 95% ethanol. After incubation for 20-30 minutes in dark, the PCR product then centrifuged at 13000 rpm for 25-30 minutes. Removed the supernatant gently with micro-pipette and re-suspended the pellet with 70% ethanol and then centrifugation for 10 minutes. Again removed the supernatant after centrifugation with micropipette and dry the pellet in air for about 30 minutes. Air dried pellet was re-suspended with 15µl formamide. Before submission to sequencer, the samples were heat shocked at 95^{o}C for 5 minutes then transferred on ice immediately.

iv) Sample submission for loading on ABI PRISM 3700 Sequencer

DNA sequencing was performed by using ABI 3700 genetic analyzer. Sequence Navigator tool was to fetch sequence from the given fluorimetric scan data. This sequence was further assembled for proper insight of the sequencing data. CHROMAS version 2.0, a sequence analysing software tool, was used to analyse

the sequencing information before aligning with sequences present in NCBI database representing *PVY-CP* gene.

3.5. Study of sequence homology

Standard BLAST software available at NCBI website was used for homology studies (Altschul *et at.*1997*).* Clustal W were used for multiple sequence alignment4.0 by Tamura *et al.*, 2007.

3.6. *PVY*-Coat Protein (*CP*) Modeling

Expert Protein Analysis System (ExPaSy) was applied to convert the DNA sequence into protein amino acid by prediction. In 6 frame results, we selected first 5‟-3‟ frame protein sequence with lowest number of stop codons and position of stop codon in this sequence at 3‟ end.

RESULTS

In this chapter, symtomology of the *potato virus Y* and its detection through RT-PCR based modern molecular technique was described. Frequency of *potato virus Y* in selected locations was also studied.

4.1. Identification of *potato virus Y* on the basis of characteristics symptoms

Stunting, systemic vein clearing, mosaic, mottling, curling, shortening of leaves, dark green and vein-banding were observed in the leaves of the infected potato plants. In few plants with systemic vein clearing and vein-banding were found.

Figure 10. Typical symptoms of mosaic of potato caused by *PVY*

4.2. Incidence (%) of *PVY*

Symptoms of *PVY* that appear at investigated areas and its relative incidence level in random samples were estimated. In all investigated areas, incidence of *PVY* appeared in severe to moderate level and their percent disease incidence was ranged 3-17%. In this study, the source of potato seed tubers was also studied

because this virus transmitted through insect vector aphids, mechanically and as well as also infected seed tubers. From the study, it was noticed that in most of the cases farmers of selected areas were continuously cultivating same field and used their own seed tubers that was kept in cold storage condition and frequency was 67.6% which was the highest among the all sources. The second source of potato seed tubers was government organization (BADC) and frequency was 15.5%. Others sources of potato seed tubers were local market, home storage, company seed, NGO supply and their frequency was 8.5, 5.6, 1.4, 1.4% respectively (Fig. 12).

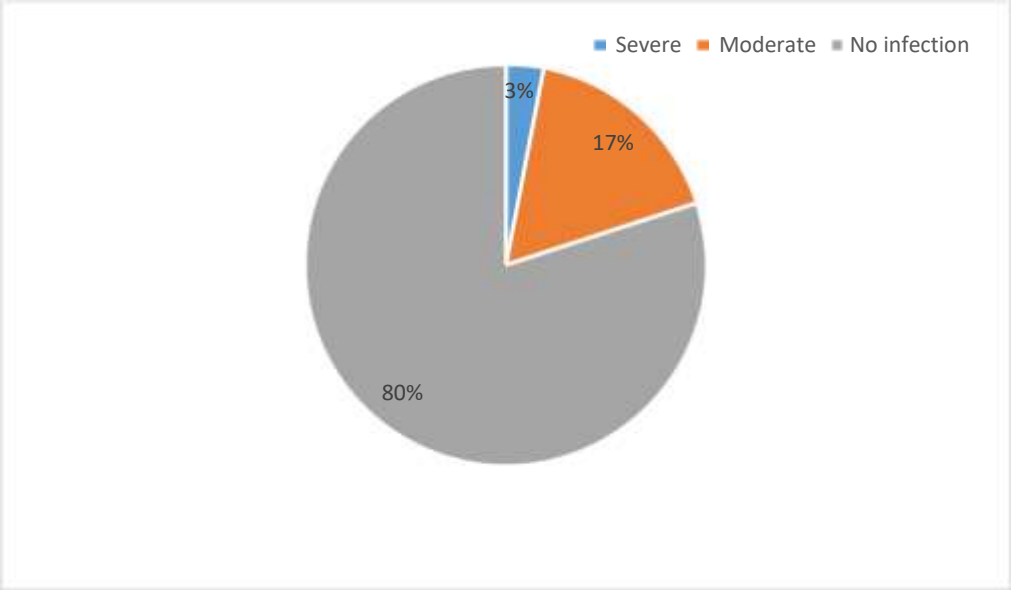

Figure 11. Incidence of *PVY* in different growing stages

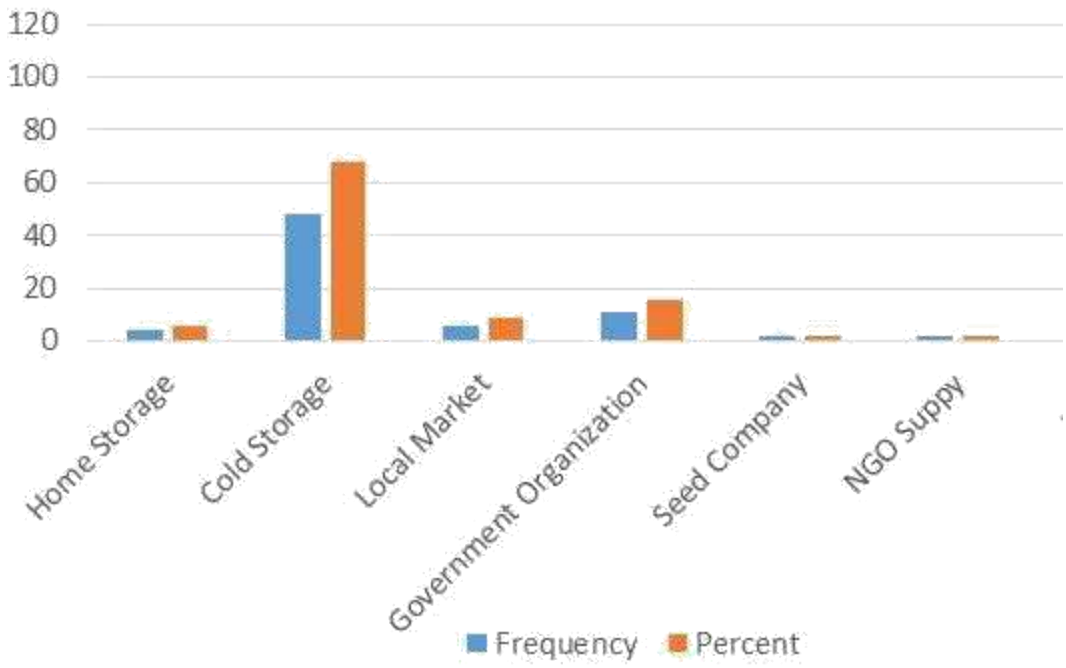

Figure 12. Source of potato tubers used in potato cultivation in 2018-2019 at selected areas

4.3. Multiple Sequences Alignments of *PVY-CP* gene

Multiple sequence alignment of all four reported *PVY* strains namely *PVY* ^{NTN} (Accession # GU550076), *PVY* ^{N} (Accession # AJ890342), *PVY* ^{O} (Accession # AJ890349) and *PVY*^{C} (Accession # AJ890348) was done using Clustal W software. Multiple sequences alignments is presented as figure 13.

```
NTN             GCAAATGACACAATTGATGCAGGAGGAAGCAGTAAGAAAGATGCAAGACCAGAGCAAGGC 60
o               -CAAATGACACAATCGATGCAGGAGAAAGCAGCAAGAAAGATGCAAAACCAGAGCAAGGC 59
C               GTAAATGAAACAATCGATGCAGGAGGGAGCAGCAAGAAAGATGCGAGACCAGAACAGGGT 60
N               -GAAATGACACAATTGATGCAGGAGGAAGCACTAAGAAGGATGCAAAACAAGAGCTAGGT 59

NTN             AGCATCCAGTCAAACCCGAACAAAGGAAAAGATAAGGATGTGAATGCTGGTACATCTGGG 120
o               AGCATCCAGTTAAACCCGAACAAAGGAAAAGATAAGGATGTGAATGCTGGTACATCTGGG 119
C               AGCATCCAGTCAAATCCTAACAAGGGAAAGGATAAGGACGTAAATGTTGGTACATCTGGA 120
```

```
N               AGCATTCAACCAAATCTCAACAAGGAAAAGGAAAAGGACGTGAATGTTGGAACATCTGGA 119


NTN             ACACACACTGTGCCGAGAATCAAGGCTATCACGTCCAAAATGAGAATGCCCAAAAGTAAG    180
o               ACACATACTGTACCGAGAATCAAGGCTATCACGTCCAAAATGAGAATGCCCAAAAGCAAG    179
C               ACACATACTGTACCAAGAATAAAGGCGATTACGTCCAAAATGAGAATGCCTCAAAGCAAG    180
N               ACTCATACTGTGCCACGAATTAAAGCTATCACGTCCAAAATGAGAATGCCCAAGAGTAAA 179

NTN             GGAGCAACCGTGCTAAACTTAGAACACTTGCTTGAGTATGCTCCACAACAAATTGATATT    240
o               GGAGCAACCGTGCTAAACTTAGAACACTTGCTTGAGTACGCTCCACAACAAATTGATATT    239
C               GGGGCGACCGTGCTAAACTTGGAACACTTGCTCGAGTATGCTCCACAGCAAATTGATATC    240
N               GGTGCAACTGTACTAAATTTGGAACACTTACTCGAGTATGCTCCACAGCAAATTGACATC 239


NTN             TCAAATACTCGGGCAACTCAATCACAGTTTGATACGTGGTATGAGGCAGTGCGGATGGCA 300
o               TCAAATACTCGGGCAACTCAATCACAGTTTGATACGTGGTATGAGGCAGTGCGGATGGCA 299
C               TCAAATACTCGGGCAACTCAATCACAGTTTGATACATGGTATGAAGCAGTACGGGTGGCG 300
N               TCAAATACTCGAGCAACTCAATCACAGTTTGATACGTGGTATGAAGCGGTACAACTTGCA 299


NTN             TACGACATAGGAGAAACTGAGATGCCAACTGTGATGAATGGGCTTATGGTTTGGTGCATT 360
o               TACGACATAGGAGAAACTGAGATGCCAACTGTGATGAATGGGCTTATGGTTTGGTGCATT 359
C               TACGACATAGGGGAAACTGAAATGCCAACTGTGATGAATGGGCTTATGGTTTGGTGCATT 360
N               TACGGCATAGGAGAAACTGAAATGCCAACTGTGATGAATGGGCTTATGGTTTGGTGCATT 359


NTN             GAAAATGGAACCTCGCCAAATGTCAACGGAGTTTGGGTTATGATGGATGGGAATGAACAA 420
o               GAAAATGGAACCTCGCCAAATGTCAACGGAGTTTGGGTTATGATGGATGGGAATGAACAA 419
C               GAGAATGGAACCTCGCCAAATATCAACGGAGTTTGGGTTATGATGGATGGAAATGAACAA 420
N               GAAAATGGAACCTCGCCAAACATCAACGGAGTTTGGGTTATGATGGATGGAGATGAACAA 419
```

```
NTN         GTCGAGTACCCGTTGAAACCAATCGTTGAGAATGCAAAACCAACCCTTAGGCAAATCATG 480
o           GTTGAGTACCCGTTGAAACCAATCGTTGAGAATGCAAAACCAACCCTGAGGCAAATCATG 479
C           GTTGAATACCCGTTGAAACCAATCGTTGAGAATGCAAAACCAACCCTTAGGCAAATCATG 480
N           GTCGAATACCCACTGAAACCAATCGTTGAGAATGCAAAACCAACACTTAGGCAAATCATG 479

NTN         GCACATTTCTCAGATGTTGCAGAAGCGTATATAGAAATGCGCAACAAAAAGGAACCATAT 540
Q           GCACATTTCTCAGATGTTGCAGAAGCGTATATAGAAATGCGCAACAAAAAGGAACCATAT 539
C           GCACATTTCTCAGATGTTGCAGAAGCGTATATAGAAATGCGCAACCAAAAGGAACCATAT 540
N           GCACATTTCTCAGATGTTGCAGAAGCGTATATAGAAATGCGCAACAAAAAGGAACCATAT 539
            ************************************************ ***************

NTN         ATGCCACGATATGGTTTAATTCGAAATCTGCGGGATGAGGGTTTAGCGCGTTATGCCTTT 600
Q           ATGCCACGATATGGGTTAATTCGAAATCTGCGGGATGTGGGTTTAGCGCGTTATGCCTTT 599
C           ATGCCACGATATGGTTTAAATCGAAATTTGCGGGATGGAAGTTTGGCGCGCTATGCCTTT 600
N           ATGCCACGATATGGTTTAGTTCGTAATCTGCGCGATGGAAGTTTGGCTCGCTATGCTTTT 599
            *************** *** *** *** **** **** **** ** ** ***** ***

NTN         GACTTTTATGAGGTCACATCACGAACACCAGTGAGGGCTAGGGAAGCGCACATTCAAATG 660
Q           GACTTTTATGAGGTCACATCACGAACACCAGTGAGGGCTAGGGAAGCGCACATTCAAATG 659
C           GACTTTTATGAAGTCACATCACGAACACCAGTGAGGGCTAGGGAAGCGCACATACAAATG 660
N           GACTTTTATGAGGTCACATCACGAACACCAGTGAGGGCTAGGGAAGCGCACATTCAAATG 659
            *********** ********************************************** ******
```

Figure 13. Multiple sequence alignment results done among four reported *PVY* strains and sequences were taken from NCBI GenBank

4.4. RNA Extraction

PVY infected potato plants were selected on the basis of typical symptoms and severe mosaic appearing leaves were used for RNA extraction. Total RNA was extracted from *PVY* +ve leaves following the "single-step" method as described in methodology section. This RNA was used for cDNA synthesis, before cDNA synthesis RNA was quantified in 1% agarose gel and analyzed results is presented in Figure 14.

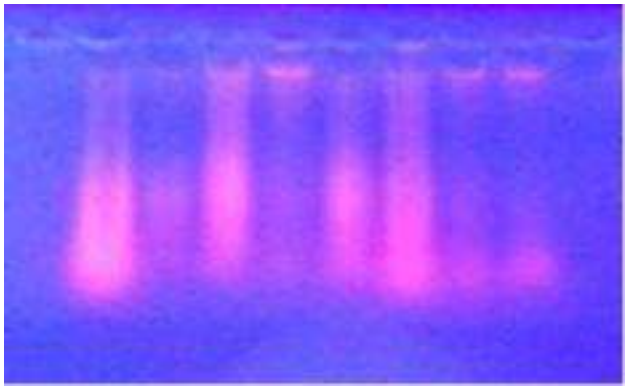

Figure 14. Total RNA from infected leaves of potato analyzed at 1% agarose gel

4.5. Amplification of *PVY -CP* gene

cDNA was synthesized from extracted RNA that was used as a template in RT-PCR amplification, using gene specific primers, designed to amplify 480 bp fragment of *PVY* from the coat protein gene region. Total volumes of RT-PCR products were resolved on 1.5% agarose gel along with 50 bp DNA ladder. The expected size of the PCR product was ~ 480 bp as clearly depicted in figure 15.

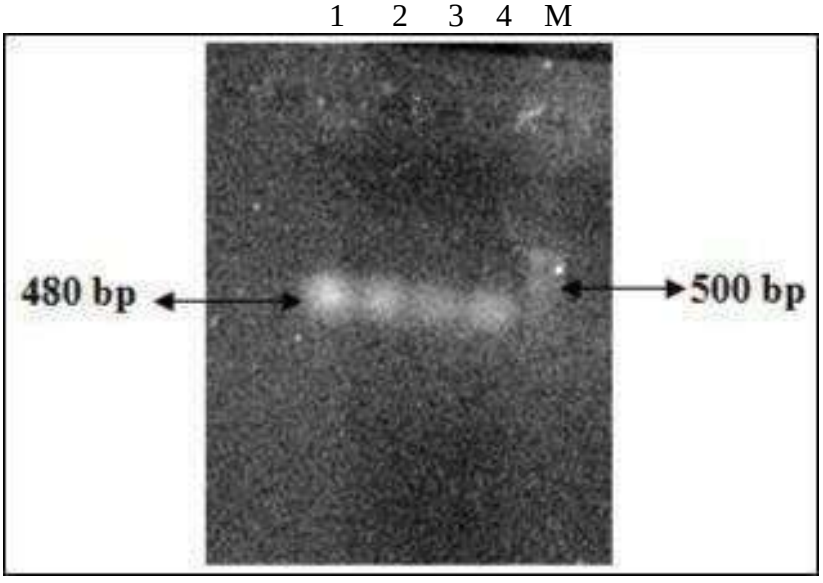

**Figure 15. A product of *PVY- CP* gene amplified at 480 bp.
Where lane 1-4 are amplified band and
M-50 bp DNA ladder.**

4.6. Sequencing PCR

For the sequencing, the *PVY-CP* amplified fragment, PCR product was used as a template with gene specific forward or reverse primer. *PVY-CP* amplified gene was sequenced through automated DNA sequencing system. Nucleotide sequence results of *PVY-CP* gene was aligned with genes of other isolates of *PVY* reported in GenBank database.

4.7. Sequence Analysis

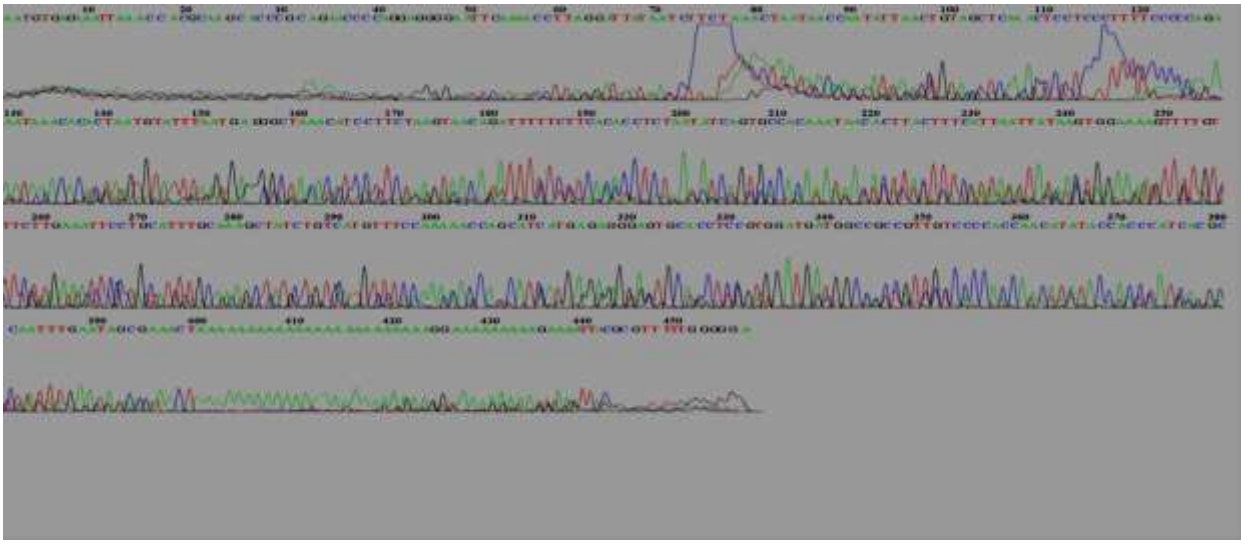

Figure 16. Fluorimetric scans from the sequence (forward) that was assembled by using the sequence Navigator software

AATGTGAGAAATTAAACCACGCAAGCACCCGCAGAACCCCAGGAGGGGAAT
TCAAAACCTTAGGATTATAATCTTCTAAACTAATAACCAATATTAACTGTAGC
TCAAACTCCTCCCTTTTCCCCCAGAAATAAACACACTAATGTATTTAATGAGG
GCTAAACATCCTTCTAAGTAACAGATTTTTCTTCACACCTCTAATATCAGTGC
CACAAATAACACTTACTTTCATTAATTATAAGTGGAAAAGTTTTGTTTCTTGA
AATTCCTGCATTTGCAAAGCTATCTGTCATGTTTCCAAAAACCAGCATCATGA
GAGGGAGTGCACCTCCGTGGATGATGGCCGCCGTTGTCCCCACCAACATATA
CCACCCATCACGCCAATTTGAATAGCGAAACTAAAAAAAAAAAAAAAAAAAA
AAAAAGGAAAAAAAAAGAAAATTACGCGTTTTTGGGGGAATGTGAGAAATT
AAACCACGCT

Figure 17. Sanger dideoxy sequencing of *PVY-CP* gene (FASTA nucleotide sequence, 480 bp, forward).

4.8. Homology Study

The comparison of the sequenced gene showed 96-98% homology of this isolated sequence was obtained with 100 reported *PVY* sequences of NCBI. Australian isolates "Accession no. KP691324.1 and JN034576.1" showed 96% homology and 98% homology was found in Iran isolates, "Accession no. EF192313.1", European isolates "Accession no. KP063209.1" showed 98% homology and with China isolate "Accession no. KU375553.1" showed 97% homology.India isolates "accession no. JQ034576.1" showed 97% homology with newly sequenced PVY-CP gene.

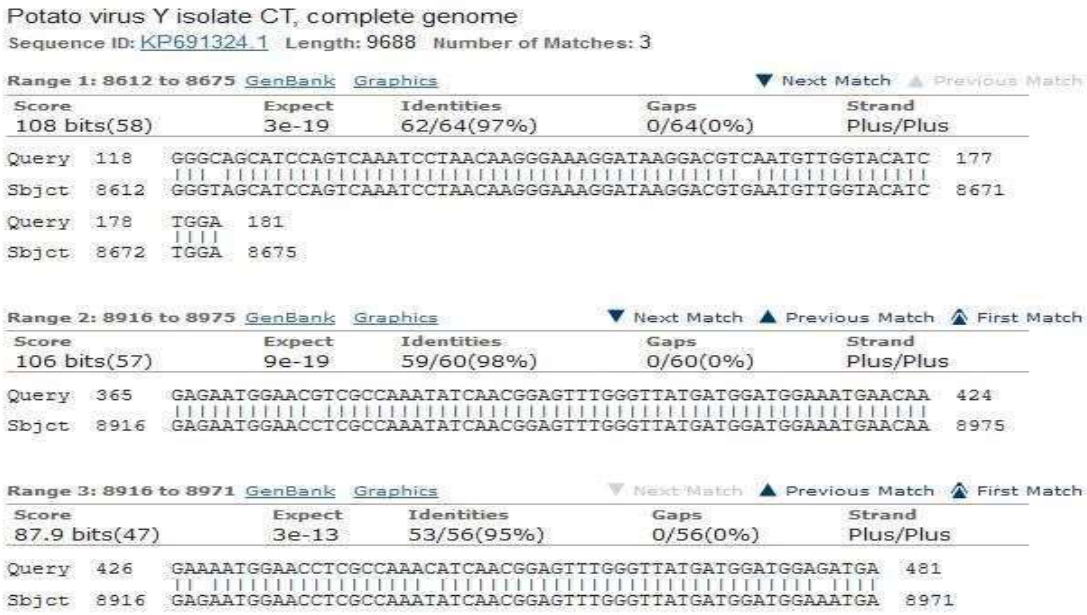

Figure 18. Complete genome sequence of *PVY* isolate CT(Accession no. KP063209.1)

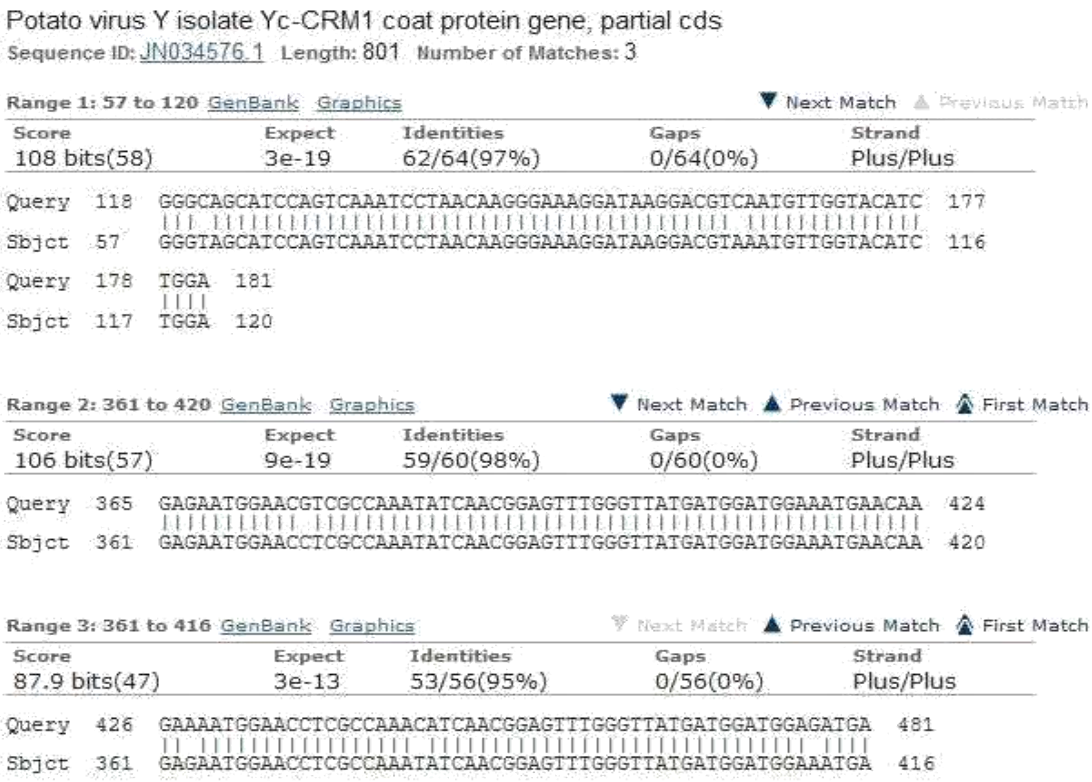

Figure 19. Complete genome sequence of *PVY* isolate Y_C - RM1(Accession no. JN034576.1)

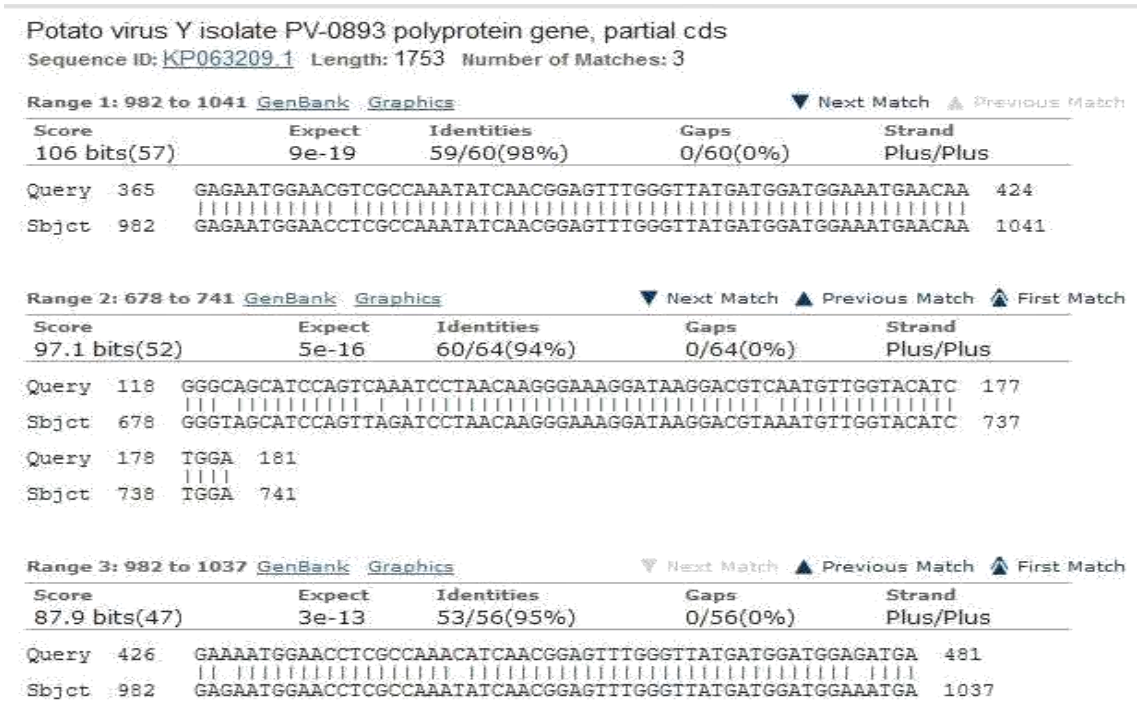

Figure 20. Complete genome sequence of *PVY* isolate PV-0893 polyprotein gene(Accession no. KP063209.1)

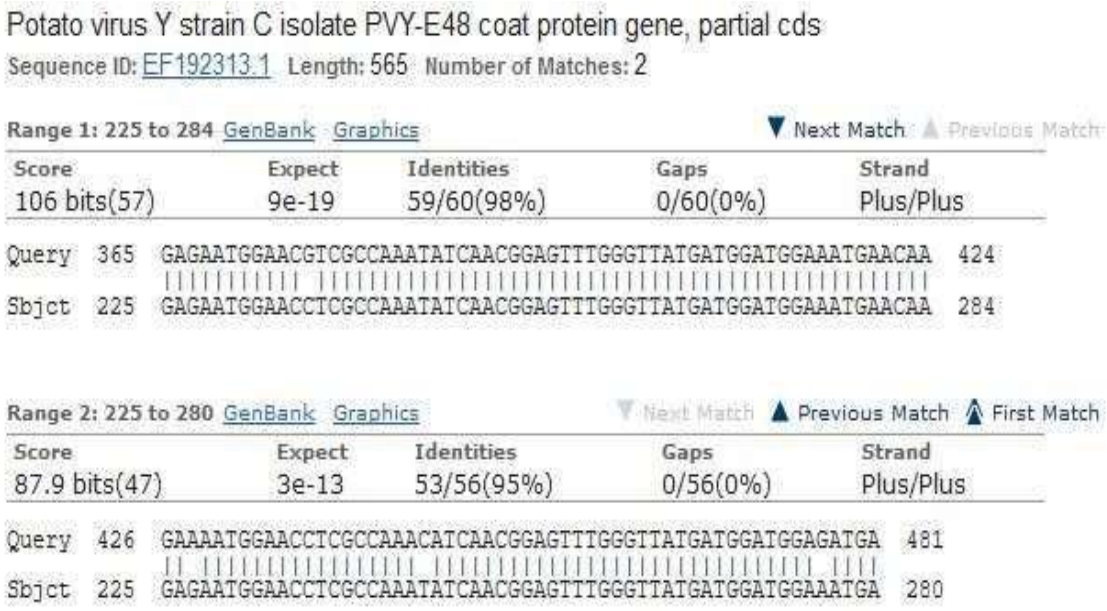

Figure 21. Complete genome sequence of *PVY* isolate *PVY*-E48 coat protein gene(Accession no. KP063209.1)

4.9. Predicted Protein Sequence

Predicted protein sequence *of* amplified fragment of *PVY-coat protein* (*CP*) gene was revealed through ExPaSy Protein Translate software. The predicted protein sequence is given below as figure 22.

5'-3' Frame

ASSQTRTKVTIRMLVHLGASSTRTKVKIRMLVHLGSIQSN
PNKGKDKDVNVGTSGXHSTKSQHGKGKGRECWNIWRNW
NLAKCQRSFGYDGWETRQWNLAKCQRRLGYRWMGMNN
ENGTSPNINGVWVMDGNEQXKWNLAKHQRSLGYDGWR

Figure 22. Predicted protein sequence of 480 bp amplified fragment of *PVY-CP* gene

DISCUSSION

Three selected potato growing areas of Bangladesh were surveyed viz. Munshiganj, Manikganj and savar and data were collected on typical symptoms of potato virus Y diseases. Typical symptoms of potato virus Y diseases is yellowish-green mosaic pattern on leaves and stunted growth of plant. Souza Dias and Iamauti (1997) described that symptoms of *PVY* infection in potato include mosaic patterns on leaves, leaf distortion, brown or black(necrotic) line patterns often on veins or shoots, stunted growth, death of growing points, and tuber necrosis.

4.10. Disease Incidence

The incidence of potato viruses like *PLRV, PVY* and *PVX* are major constraint in potato production worldwide and also in Bangladesh. In all investigated areas, *PVY* appeared in severe to moderate level and their % incidence was 3-17%. Hossain et al., 2019 narrated that PLRV, *PVY*, and PVX are the severely affecting potato viruses that reduce potato yields by 10-90%. There was a significant difference in the infection percent among the different locations. The highest infection percentage was in the locations that belong to savar and Manikganj.While in other location such as Munshiganj low infection was detected in the screened locations. Many factors play a crucial role in infection variation of *PVY* incidence. Of these, cultivation history, cultivation practices, preventive measurements and temperature are the main ones. Temperature is very important for the virus activity and multiplication. Zheng et al., (2005) narrated that the largest number of plants became infected when *PVY* were appeared at 20°C and low above 30°C. Moreover, these samples were that collected during the fall growing season of 2018-19, and the temperature in this season is very suitable for the virus activity and multiplication. As the temperature was higher in Munsigonj than others district which is important factors for the

virus transmission through the insect vectors thats because low infection was appeared in Munshiganj. This may indicate that the prevalence of potato viruses is not consistent from one location to another. Thus, it would be useful to perform further studies on potato viruses‟ prevalence in the main growing regions of potatoes in Bangladesh. Such information would also be useful in developing new strategies for control of viruses occurring in main potato growing regions.

4.11. Molecular Detection

To manage the viral diseases, it is important to detect the viruses. There are various methods of detecting viruses such as, Biological methods, Physical methods, Physio-Chemical methods, viral coat protein methods, Modern Molecular Techniques. Viral diseases can be detected biologically through symptoms, Transmission methods and host range. These methods are not reliable to detect viruses. Physical methods are also used to detect viruses through Thermal inactivation point (TIP), Dilution end point (DEP), Longevity *in-vitro* (LIV). But these properties are unreliable and no longer recommended for virus diagnosis. Viruses are also detected by Particle size and Morphology, Buoyant density (partial volumic mass) Sedimention coefficient. Among the above mentioned methods, Electron microscopy (EM) provides very useful information on the morphology of the virus particles and is commonly used for virus detection when EM facilities are readily available. For example: Filamentous and rod-shaped viruses such as potyviruses, potexviruses, and tobamoviruses can more readily be differentiated in negatively stained leaf-dip preparations than isometric viruses and other viruses. But the viruses that occur in low concentrations in plant sap like *PVY*, are not easily seen unless the virus in the test material is concentrated before visualization. The efficiency of virus visualization can be improved in combination with serology. On the other hands, EM is labor intensive and expensive, it cannot often be used for the rapid processing of multiple samples.

Many agricultural research institutions cannot afford to have an electron microscope facility due to the prohibitively high costs involved in installation and maintenance of the facility. Modern molecular techniques are more reliable to detect the potyviruses. Viral Coat Protein based Method such as DAS-ELISA is reliable but to detect the *PVY,* RT-PCR is more reliable, robust and highly sensitive in a short time (Schoen *et al.,* 1996).

4.12. RT-PCR amplification

Collected samples were screened by using molecular test like RT-PCR is used to diagnose potato virus. This technique successfully detected the virus from potato leaf samples even in the leaves that were asymptomatic and RT-PCR is more reliable and sensitive with added advantage of less time involved than other serological tests. Saiki *et al.,* 1985 described that successful PCR reaction is because of the choice, quality and accuracy of various factors like template DNA/cDNA (RT-PCR), primers, dNTPs, concentration of magnesium ion, choice of polymerase enzyme and primer annealing temperature. RT-PCR gives higher sensitivity with high speed diagnosis and reduced sample size, therefore, it was found good alternative to other diagnostic method like ELISA. Schoen *et al.,* (1996) suggested that, as a reliable and effective test, the RT-PCR method should be used for routine diagnosis of potato viruses because it showed higher sensitivity.

SUMMARY AND CONCLUSIONS

Potato is a one of the most popular crop in Bangladesh. *Potato virus Y (PVY)* is one of the most common virus that infected potatoes and reduced the yield in potato growing areas. In this study, samples were collected from selected potato growing areas of Bangladesh viz Savar, Manikganj and Munshiganj. Polymerase chain reaction (PCR) is an extremely specific, sensitive and versatile method that have a great potential to amplify trace amounts of targeted nucleic acid using specific primers to the region of amplification, and thermostable DNA polymerase. *PVY* was detected through Reverse Transcription Polymerase Chain Reaction (RT-PCR) which offers a potentially more sensitive method for detection of viruses from plant leaf samples. In this study, *PVY* was detected through RT-PCR which is one of the reliable, robust, and highly sensitive method for molecular detection of *PVY*. The study provides a platform for *PVY* diagnose that will be helpful to create proper management approaches to manage or control *PVY* diseases in Bangladesh. Although PCR can achieve a high sensitivity and specificity, different comparative assays a failure of PCR amplification to correctly diagnose infected and non- infected plant material has been reported. In this investigation, considering the incidence level of potato virus in the field conditions it may be concluded that three (3) major potato viruses viz. *Potato leaf roll virus (PLRV), Potato virus Y (PVY*) and *Potato virus X (PVX)* were common in all major potato growing areas of Bangladesh. Moreover, in this study virus *PVY* was detected through molecular technique (RT-PCR), this technique also needs to perform for detection of other existing potato viruses in Bangladesh.

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APPENDICES

Appendix-I

Preparation of 5X TBE buffer

Tris-Cl-0.7g

Boric acid-13.75g

EDTA-2.325g

Distilled water upto 500 ml

pH 8.0

Appendix-II

Preparation of 1X TBE buffer

5X TBE buffer-100ml

Distilled water - 400 ml

Appendix-III

Preparation of Stock solution

Stock solution (Forward primer): Forward primer + 532 µl DEPC treated water

Stock solution (Reverse primer): Reverse primer + 392 µl DEPC treated water

Appendix-IV

Preparation of working solution

10µl Forward primer + 90 µl DEPC treated water =100 µl (10 pmoles)

10µl Reverse primer + 90 µl DEPC treated water =100 µl (10 pmoles)

Appendix-V

cDNA Synthesis

RNA-4 µl

Forward/ Reverse primer -1 µl

Nucleus free water-6 µl

Appendix-VI

Preparation of 100 ml Gel Preparation (1% gel)

1x TBE Buffer-100ml

Agarose gel-1000 mg

Heat for 3 minutes

After cooling, Ethidium bromide- 2.31 µl

Appendix-VII

Preparation of 6X DNA Loading Dye

Bromophenol blue - 20 µl

DEPC treated water - 60 µl

Appendix-VIII

Gel Electrophoresis

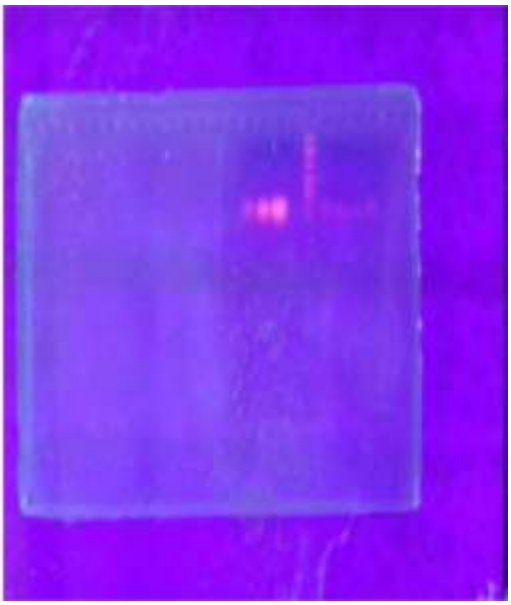
Sample-2 µl

Loading Dye-2 µl

Appendix-IX

Lab Work

Loading Dye



Gel electrophoresis **DNA band under UV light**

Appendix-X- Structured questionnaire

Title of the Project

Incidence of *Potato Virus Y* (*PVY*) in Selected Districts of Bangladesh and It's Molecular Detection

"Questionnaire for potato cultivars to investigate the incidence of *potato virus y* in Bangladesh''

1. Information of DAE personnel at upazilla level:

Name:……

Designation:……

Address:……

Cell No:

2.0. Information of growers/farmers:

2.1 Name:……

2.2 Gender: Male Female

2.3 Age: (Code:1 =20 or less,2=21to30,3=31 to 40,4=41to50, 5=51 or above

2.4 Educational background:(Code: 1=5to8, 2=8toSSC, 3=HSC, 4= Graduate

2.5 Area cultivated:……..

2.6 Previous crops history of selected area (Potato cultivated or not?) YES NO

2.7 Communication with others organization: YES NO

3.0. Information about potato seed tubers:

3.1. Source of seed tubers:

a) Home storage
b) Clod storage
c) Local market:
d) Improve farmer
e) Govt. agencies
f) Company seeds
g) NGO"s supply

3.2. If source of seed tubers from company then please be specific ----------

3.3. Information about seed tuber treatments:

a) Hot water treatment ☐
b) Treated with ash ☐
c) By using fungicides ☐
d) Other: ☐

3.4. Name of the fungicides used for seed tubers treatment:

a) MEMC ☐
b) Boric acid ☐
c) Bavistin ☐
d) Other ☐

4.0. Information about potato varieties:

4.1 Name of the potato varieties used:

a) Chardinal. ☐
b) Diament. ☐
c) Chamak. ☐
d) Lalpakhri. ☐
f) Gronal ☐
g) Hira ☐
h) Other ☐

4.2. Mostly cultivated varieties:

a) Local ☐
b) Improve ☐
varieties c) Hybrids ☐

4.3. Date of sowing:

5.0. Information about disease incidence of major potato viruses:

5.1. Major potato viruses symptoms that appear at investigated area:

a) *PLRV*
b) *PVY* ☐
c) *PVX* ☐
d) other ☐

5.2. Incidence of *PLRV* on the basis of typical morphological symptoms:

a) Severe / Epidemic form
b) Moderate
c) Low infection
d) No-infection

5.3. Incidence of *PVY*

a) Severe / Epidemic form
b) Moderate
c) Low infection
d) No-infection

5.4. Incidence of *PVX*

a) Severe / Epidemic form
b) Moderate
c) Low infection
d) No-infection

5.5. Status of insect vectors:

a) Several numbers
b) Moderate numbers
c) Few numbers
d) No insect vectors present

5.6. Used of insecticides to control the insect vectors:

a) Epidacloromite
b) Monochotophos
c) Dimethoate
d) Pirimicarb
e) Pymetrozine
f) Others.

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Signature of Farmer/Grower Signature of Research Assistant

Signature of Research Coordinator