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MOLECULAR IDENTIFICATION OF THE POTATO VIRUS M ISOLATE PVM-UZ AY1 WITH COAT PROTEIN (CP) GENE AND PHYLOGENETIC ANALYSIS

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SUMMARY

Potato virus M (PVM) is one of the most widespread and economically damaging viruses worldwide, along with X, Y, S, A, and L. Therefore, outcomes of the monitoring surveys carried out in potato fields during this study showed numerous disease symptoms visible on potato plants, such as boat-shaped curling of leaf tips, mosaic twisting, leaf swelling, striped mosaic along the veins, and spotted mosaic. The diseased sample collections from potato plants and the molecular and genetic identification progressed based on the PVM protein coat (CP) gene. The results revealed that the boat-shaped curling and mosaic symptoms were PVM's typical signs. The isolates' identification continued through PCR product sequencing, and their submission in the NCBI database received a PP235793.1 tag and the name PVM-Uz-AY1. The phylogenetic analysis of the identified isolates showed that the said isolate was 98% related to the isolate GQ469609.1, and the rest to isolates, such as VF-Nevsky2 (LC511899.1), FE-Queen_anne10 (LC511893.1), and KC479342.1. Those located on other phylogenetic branches were KF561611.1, KF561603.1, and KF561608.1, which appeared to share 99% homology. In addition, the study identified the virus using the Real Time-PCR method, obtaining reliable results.

Keywords: Potato (*Solanum tuberosum* L.), PVM, isolate, RT-PCR, CP gene, PVM-Uz-AY1, primer, phylogenic analysis, Uzbekistan

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Key findings: PVM-Uz-AY1 isolate of the PVM infecting potato (*Solanum tuberosum* L.) succeeded in identification using RT-PCR. The nucleotide sequence study of the CP gene responsible for this virus' protein coat was intensive. The construction of a phylogenetic tree of the PVM-Uz-AY1 isolate occurred based on the bioinformatic analysis, determining the evolutionary origin of the virus.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is the most valuable crop consumed by the population and grown worldwide. According to the United Nations Food and Agriculture Organization (FAO), worldwide potato production reached approximately 375 million tons in 2022 (FAOSTAT, 2023). According to the State Statistics Committee of Uzbekistan, more than 3,400 tons of potatoes produced in the Republic emerged during 2022, and about 3,740 tons in 2023, with 92.0% of total potatoes grown from the farming community. Additionally, potato importation reached 478,300 tons from 16 countries to meet the population needs (Jovlieva *et al.,* 2024).

Therefore, developing productive potato cultivars in Uzbekistan, identifying various diseases, and reducing their damage to productivity are dire requirements with immense practical importance. Like other crop plants, many pathogens infect the potato plant, including fungi, bacteria, mycoplasmas, viruses, viroids, and nematodes, reducing yield and quality. Among them, along with phytophthora (*Phytophthora infestans* [Mont.] de-Bary), the viruses are the most crucial pathogens (Ahmad *et al.,* 2012; Adolf *et al.,* 2020; Matniyazova *et al.,* 2022).

Many reports stated more than 50 viruses have infected potato plants worldwide, among which Y, S, A, X, and L are usually common (Acosta-Leal *et al.,* 2011), with PVM as one such virus (Adams and Antoniw*,* 2004). The first information about the spread of this virus surfaced in 2011 in Uzbekistan (Fayziev *et al.,* 2020). PVM is one of the most widespread and genetically diverse viruses globally (Kowalska and Was, 1976; Khurana and Singh*,* 1980; Cavileer *et al.,* 1998; Glasa *et al.,* 2019; Halabi *et al.,* 2021; https://www.dpvweb.net/dpv/showdpv/?dpvno=87). In particular, Indian scientists who researched the diversity of this virus genome identified a new PVM-Del-144 strain of the virus found in this country based on the complete genome sequence (Kumar *et al.,* 2023).

This virus belongs to the family Betaflexiviridae, genus *Carlavirus*; its RNA is single-stranded, the genome consists of 8,500 pairs of nucleotides, and it affects and reduces crop productivity by 15%–45% (Xu *et al.,* 2010). Virus transmission is by mechanical inoculation and causes local and systemic spotting in *Datura metel* plants, red spot in Vigna plants, brown ring-shaped local wound in *Nicotiana debneyi*, and linear necrosis in *Solanum rostratum* stem and leaf veins. The *Chenopodium album* does not cause disease symptoms (Bagnall *et al.,* 1956; Hiruki*,* 1970). The virus transmission to the plant has a non-persistent route, i.e., by aphids, including *Myzus persicae* Sulz, and mechanical inoculation of infected leaf sap (Devaux *et al.,* 2021). A plant infected with the virus shows symptoms such as mosaic spots, twisting or curling, and slow growth. Some symptoms of PVM are similar to those of other potato viruses (PVX, PVY, PVS, and PVL) (Flatken *et al.,* 2008).

Numerous studies have investigated the molecular and genetic characterization of PVM; the length of the virion is about 600 nm to 1000 nm, and its genome consists of 8,534 nucleotide sequences, containing six open reading fragments (ORFs), each of which encodes separate proteins (Gramstat *et al.,* 1990; Zavriev *et al.,* 1991; Fujita *et al.,* 2018). In particular, the first codon, starting with 76 nucleotides, is responsible for synthesizing a 223-kDa protein, an enzyme that replicates the viral RNA. The next block consists of three ORFs that encode three polypeptides of 25 kDa, 12 kDa, and 7 kDa. The third block

consists of two ORF regions, the first of which encodes a 34-kDa polypeptide and the second an 11-kDa polypeptide, and each of them performs specific functions in the viral genome (Flatken *et al.,* 2008; Tabasinejad *et al.,* 2014; Plchova *et al.,* 2015).

Potato main viral diseases in Uzbekistan are PVX, PVY, PVS, PVL, PVM, and PVA, and in obtaining high-quality harvest, these viruses pose a negative impact (Fayziev *et al.*, 2020). In particular, the complex form of these viruses causes increased economic losses in potato fields (Awasthi and Verma*,* 2017). In recent years, the effective research on viruses infecting important crop plants has been progressive in Uzbekistan (Sobirova *et al.,* 2020, 2023; Fayziev *et al.,* 2020). It included determining the virus infection levels of plants belonging to different genotypes (Sobirova *et al.,* 2020), molecular identification of the virus (Sattorov *et al.,* 2020; Makhmudov *et al.,* 2023), obtaining specific serum for virus immunodiagnosis and its practical use (Jovlieva *et al.,* 2024), and studies on the virus effects on crop plants' physiological properties (Sobirova *et al.,* 2023).

Carrying out measures to combat viruses necessitates studying the biological characteristics, the level of spread of the virus using sensitive methods (Clark and Bar-Joseph*,* 1984), the effect of various environmental and soil factors on plants (Ramazonov *et al,* 2020), and genetic characteristics (Amanov *et al.,* 2022; Buronov *et al.,* 2023; Muminov *et al.,* 2023). However, detecting the virus in buds is crucial using fast and sensitive methods, which reduce viral spread and economic damage (Jeevalatha *et al.,* 2013; Malko *et al.,* 2019). Therefore, the latest work's chief goal was the PVM molecular identification.

MATERIALS AND METHODS

Sample collection

The KMV disease symptoms' visual manifestation in the potato cultivar 'Umid' field began in September 2023 in the Tashkent Region, Uzbekistan. The storing of samples in the Thermo Fisher Scientific (AQSH) refrigerator had a -20 °C temperature before bringing to the laboratory. The excised plant leaves in labeled plastic bags reached storage at −80 °C to prevent viral RNA degradation.

Extraction and purification of total RNA

The homogenization of 0.1 g leaf samples continued in liquid nitrogen, with the total RNA extracted according to the manufacturer's protocol using Invitrogen™ PureLink™ RNA Mini Kit (Thermo Fisher Scientific, USA). Measurement of quantity and quality of total RNA also transpired using a spectrophotometer NanoDrop Eight (Thermo Fisher Scientific, USA), storing the RNA samples at -80 °C until used for RT-PCR.

Synthesis of the cDNA

The cDNA synthesis used SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific, USA) and buffers in a final volume of 20 µl. Taking 5 ml from the total extracted RNA solution bore mixing with 1 µl of 10 µM reverse primer PVM2 (Table 1), 4 µl 2, 5mM dNTP, and 3 µl nuclease-free water. Afterward, samples reached incubation at 65 °C for 5 min and quenched on ice for 3 min. The addition of other reaction components was in the following order: 4 µl of 5X PCR buffer, 1 µl 0.1 M dithiothreitol, and 0.5 µl (20 U) of SuperScript

Table 1. Primers used in this study.

Target region	Name of the primers	Sequence (5′-3′)	Product size (bp)	Source
CP	PVM1-R	CTTCATTTGTTATTCGACTT	917	Xu *et al.,* 2010
CP	PVM1-F	ATGGGAGATTCAACRAAGAA		

IV reverse transcriptase and nuclease-free water to give a reaction volume of 20 µl. The tubes underwent incubation for 1 h 55 min at 41°C on a T960 PCR Thermal Cycler, followed by 10 min heating at 70 °C to denature the enzyme.

Amplification by PCR

Primers PVM1 (Reverse: CTTCATTTGTTATTCGACTT) and PVM2 (Forward: ATGGGAGATTCAACRAAGAA) served to amplify the entire viral coat protein (CP) gene (917 bp) (Xu *et al.,* 2010). The virus diagnostics using the Real-Time PCR method continued with the help of a diagnostic kit obtained from the company "Sintol" (Russia). PCR application employed the Platinum hot start PCR 2x master mix (Thermo Fisher Scientific, USA) in a 25 µl volume, containing 4 µl 2X Master Mix, 0.5 µl of each primer (10 µM), 0.9 µl 25 mM MgCl, and 4 µl of the cDNA. The following thermocycling program occurred for PCR: initial denaturation at 94 °C for 2 min, denaturation at 94 °C for 30 s, annealing at 50 °C for 45 s, elongation at 72 °C for 1 min - 45 cycles, and final elongation at 72 °C for 5 min (Xu *et al.,* 2010).

Electrophoresis in agarose gel

Analysis of PCR products by electrophoresis progressed on 2% agarose gel prepared in 1x tris-borate-EDTA buffer (TBE, Thermo Scientific) stained with ethidium bromide. The 2 µl of DNA Gel Loading Dye (6X) and 1.3 µL of 100 bp DNA Ladder addition to the first well of agarose gel ensued, then adding 10 µl PCR products with 3 µl of DNA Gel Loading Dye (6X) followed into agarose wells. Electrophoresis application used horizontal electrophoresis system SE-1 (Helicon, Russia) at 100V for 100 min. PCR product visualization engaged the UV light and photographed using a gel document imaging system BK-AG100 (Biobase, China).

Sequencing of PCR products

One PCR band excision using a razor blade continued to transfer to a 1.5 ml centrifuge tube and purified, following the manufacturer's instructions using the PureLink™ Quick Gel Extraction Kit (Invitrogen, USA). Cycle sequencing reaction performance used the BigDye® Terminator v 3.1 kit (Applied Biosystems, USA). Cycle sequencing reaction consists of ddH2O-3.5 µl, BigDye- 1 µL, 5x Seg.buffer-2 µl, sequencing primer - 0.5 µl, and purified PCR product-2 µl. Sequencing used the PMV1-F and PMV1-R primers. The following thermocycling program for cycle sequencing reaction continued: initial denaturation stage at 96 °C for 1 min, denaturation at 96 °C for 10 s, annealing at 50 °C for 45 s, and elongation at 60 °C for 3 min, repeated for 45 consecutive cycles. The product of the sequence reaction gained storing at 4 °C. The sequencing reaction product purification from fluorescently labeled terminator nucleotides used the Dynabeads Sequencing Clean-Up Kit method Sanger DNA sequencing (Applied Biosystems, USA).

Separation and analysis of DNA sequence reaction products by capillary gel electrophoresis with laser-induced fluorescence detection proceeded on Applied Biosystems 3500 Genetic Analyzer (Thermo Fisher Scientific). Sequence alignments and phylogenetic analyses comprised: Snap Gene 5.3.1 program to edit the raw data obtained from the DNA sequence analysis system; Nucleotide sequences examined using BLASTN program (basic local alignment search tool nucleotide- http://www.ncbi.nlm.nih.gov/ BLAST/); Multiple sequence alignments performed by ClustalW; Phylogenetic tree construction by the Neighbor-Joining (NJ) method using Kimura 2- parameter model, with statistical support executed by the bootstrap method of interior-branch test for phylogenetic trees (Kimura, 1980). Bootstrap test of phylogeny and visualization of the final phylogenetic tree utilized the MEGA11 software package using the Molecular Evolutionary Genetics Analysis version 11 (Tamura *et al*., 2021).

RESULTS

Virological monitoring surveys commenced in potato fields of the Tashkent Region. Numerous disease symptoms, such as patchy mosaic, boat-like twisting of the leaf, non-swelling of the leaf, yellowing of the leaf veins, mosaic, and twisting of the leaves, were evident in the potato field. The collected samples for each symptom, separately placed in polyethylene bags, underwent virological studies. An infectious aphid prepared and mechanically inoculated into test-indicator plants sensitive to potato viruses helped determine the level of their infectivity. As a result, the samples collected for virological research in the potato cultivar Umid and other cultivars (Figure 1) exhibited wavy curling of edges of the potato leaf plate and upward bending of the plate and signs of vascular chlorosis, showing similar symptom characteristics of PVM.

Confirming these symptoms as specific to PVM, their molecular identification used a PCR kit for the virus molecular detection (Russia, Sintol). For this purpose, the viral RNA isolation from a plant leaf sample engaged the 'FitoSorb' RN-520 'SINTOL' (Russia) reagent kit and the sequence indicated in the test instructions. The isolated virus RNA amplification utilized the Analytik Jena qTOWER3/G equipment by 'SINTOL' (Russia) 'Potato Virus M genetic material detection test kit' for Real Time-PCR, with these symptoms confirmed by the Real Time-PCR method to be specific to the PVM virus by testing.

As mentioned above, collected samples of potato plants infected with PVM showing infectious symptoms, as confirmed by the Real Time-PCR method, incurred mechanical inoculation to test-indicator plants specific for PVM, with the disease symptoms in the indicator test plants measured (Figure 2). The succeeding study scrutinized the molecular and genetic characteristics of PVM and phylogenetic analysis, requiring a primer to identify a specific section of the virus genome. In this study, PVM used the RT-PCR (reverse transcriptase) method, applying primers designed for coat protein (CP) gene identification (Figure 3).

The PCR product, excised from the gel and purified, proceeded to sequencing at the Laboratory of Molecular Biology at the Institute of Biophysics and Biochemistry, Uzbekistan. Based on the sequence results, the nucleotide sequence obtained validation using the NCBI BLAST database. The sequence was valid to belong to PVM and accordingly enlisted in the NCBI database under the name PVM-Uz-AY1 with the identification number PP235793.1 (*http*://*www*.*ncbi*.*nlm*.*nih*.*gov/genbank)*.

This sequence product served as the basis for developing the phylogenetic tree of the PVM-Uz-AY1 isolate. The identified isolate's comparison with other isolates in the NCBI database depended on its nucleotide sequence, incurred bioinformatic analysis, and its phylogenetic tree developed using the MEGA11 program (Figure 4). As a result, the relationship of the virus isolate with the isolates identified from other regions was notable, allowing one to determine the origin and evolution of this isolate.

DISCUSSION

Based on the presented results, among the symptoms of several diseases observed in potato fields, the wave-like curling of edges of the potato leaf plate and the upward bending of the plate and inter-chlorosis of the veins were the widely distributed symptoms in potato cultivars, such as Umid, Gala, and Picas. Facts also show the symptom was specific to PVM, as confirmed by using the Real-Time PCR method. Infection of the potato plant with many viruses leads to variations in plant symptoms and increased economic losses (Adolf *et al.,* 2020; Fayziev *et al.,* 2020). Therefore, by studying a specific virus' biological, ecological, molecular, and genetic characteristics, one can recommend distinguishing it from other infections using a pure virus (Jovlieva *et al.,* 2024).

Therefore, the Real-Time PCR method confirmed the disease symptoms of the PVM-Uz-AY1 isolate in the tested indicators. The findings also underwent comparison by symptoms in a published research work, achieving varied similarities and differences, as

Figure 1. Symptoms of PVM disease in potato (*Solanum tuberosum* L.) plant. In the picture: General appearance of a plant infected with the virus (a) and symptoms on the leaf (b).

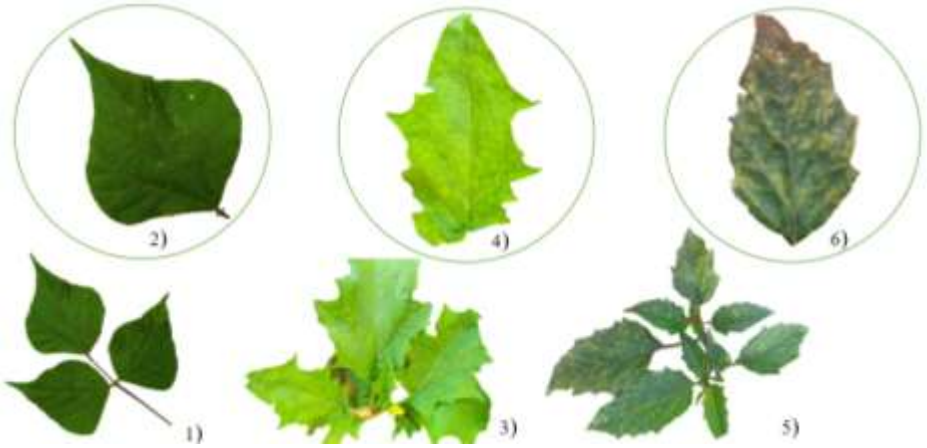

Figure 2. Disease symptoms of PVM in test-indicator plants: Symptoms on *Vigna sinensis* plant and leaf (1,2); *Datura stramonium* plant and leaf (3,4), and Phisalis plant and its leaf (5,6).

Figure 3. PVM coat protein (CP) genome RT-PCR product Electrophoresis performed in 2% agarose gel. In the picture: control 1 (virus-free potatoes), samples for RT-PCR analysis taken from Umid (2,3), Gala (4,5), and Pikas (6-8) cultivars. M - O'GeneRuler 1 kb DNA ladder (Fermentas). Primers PVM1/PVM2 RT-PCR conditions performed, as described in Xu *et al.*, 2010.

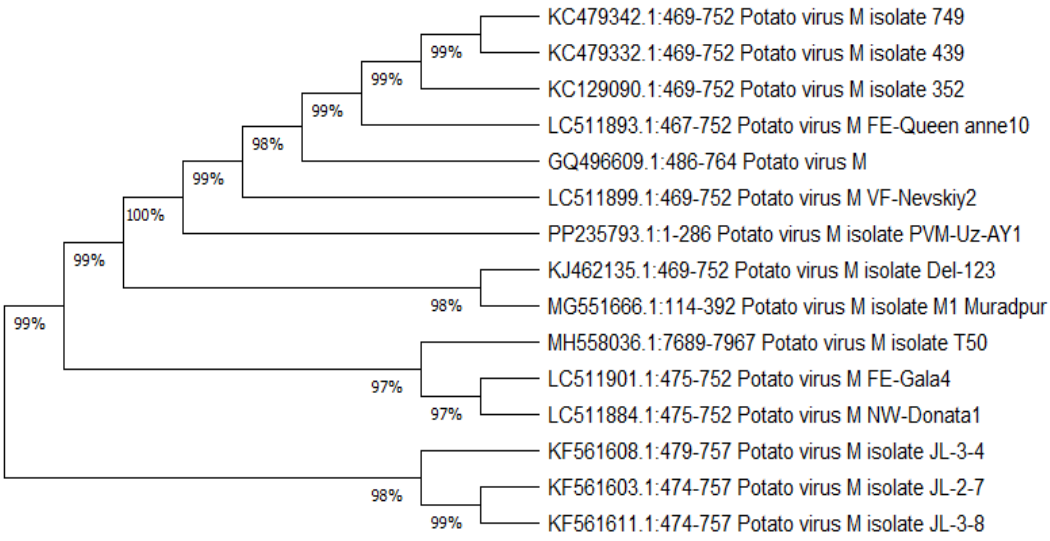

Figure 4. Phylogenetic tree of PVM-Uz-AY1 isolates.

well as the initial identification of the virus by a biological method. Consequently, the isolate of the PVM under the climatic conditions of Uzbekistan showed a yellow chloritic spot on the leaves of the *Vigna sinensis* (Figures 1 and 2). The leaf plate became pale and pointed to the system mosaic on the *Datura stramonium* (Figures 2, 3, and 4). In the Physalis plant, it appeared to cause symptoms of yellow mosaic between veins (Figure 2). Comparing these symptoms with past studies based on the biology of PVM confirmed that the symptoms were specific to this virus (Hiruki, 1970; Khurana and Singh, 1980). In turn, it was the basis for further research on the isolation of the virus, biological purification from mixed infection, multiplication, and obtaining a homogeneous pure preparation.

The RT-PCR conducted to study the molecular and genetic characteristics of the virus also showed that potato cultivars Umid, Gala, and Pikas, examined by PVM, also proved to be infected with this virus. The virus RNA isolated from the cultivar Umid also became the basis for depositing the PVM-Uz-AY1 isolate under the climatic conditions of Uzbekistan in the NCBI database for the first time. Past studies also provided the initial information about the virus spread in the climatic conditions of Uzbekistan (Fayziev *et al.,* 2020). Furthermore, information about its economic damage is available in the studies conducted in recent years (Yusubakhmedov and Fayziev, 2022).

By conducting the bioinformatic analysis of the virus and the development of its phylogenetic tree, the isolates with 98%–99% analogy formed the four separate clusters. The PVM-Uz-AY1 isolate belonged to the fourth cluster and appeared in a separate phylogenetic branch. The phylogenetic tree of isolate PVM-Uz-AY1 based on nucleotide sequence was also 98% identical to the isolate GQ469609.1. The rest of the isolates, such as VF-Nevsky2 (LC511899.1), FE-Queen_anne10 (LC511893.1), KC479342.1, and others have 99% homology with isolates, such as KF561611.1, KF561603.1, and KF561608.1 located in three phylogenetic branches (Figure 4). In turn, it indicates that the origin and evolution of the PVM-Uz-AY1 isolate were close to these isolates. The formation of virus isolates depends on the habitat, environmental conditions, and host organisms (Awasthi and Verma, 2017). A phylogenetic tree helped assess the proximity of the PVM-Uz-AY1 isolate to isolates identified in Russia. Considering that Russia is the fourth largest exporter of potatoes to Uzbekistan, one can assume that the country got the isolated virus from Russia.

CONCLUSIONS

Molecular identification confirmed that the wavy curling of the leaf plate edges, the boat-like bending of the plate up, and the symptoms of vascular chlorosis in potato plants were characteristics of PVM, with the disease test-indicator symptoms also studied in potato plants. A new Uzbek isolate, PVM-Uz-AY1, surfaced by analyzing the nucleotide sequence of the PVM CP gene isolated from the cultivar Umid using the RT-PCR method. Its consequent enlistment in the NCBI database garnered the number PP235793.1. The determined nucleotide sequence resulted in constructing a phylogenetic tree of the new isolates, distinguishing its relationship with other isolates and its evolution.

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