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Research Article

Molecular identification of potato leaf roll virus and its impact on important nutrients in tubers

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Abstract
The Potato Leaf Roll Virus (PLRV) is one of the most devastating viruses causing severe yield losses worldwide in potatoes. Comprehensive observations were made to study the PLRV infestation in major potato-growing areas of Tashkent (Uzbekistan). In the article, disease symptoms such as lightening and roughening of the leaf color and upward twisting of the leaf plate were studied by molecular identification, and it was observed that the total protein and vitamin contents were significantly lower under diseased plants. It was found out from the studied literature and conducted experiments that PLRV is mainly stored in tubers and that the stored virus affects the vitamin and total protein content of potatoes. To prevent the spread of this virus, it is essential to identify the natural reservoir plants, prevent them from growing in the fields, and plant virus-free potato varieties.
Keywords: Potato leaf roll virus (PLRV), potato, cultivar, primer, protein, vitamin

Introduction
Potato (*Solanum tuberosum* L.) is the most important crop consumed by the population and is widely grown in the world. According to the Food and Agriculture Organization of the United Nations (FAO), approximately 375 million tons of potatoes were produced worldwide in 2022 (FAOSTAT, 2023). According to the State Statistics Committee of Uzbekistan, more than 3.4 thousand tons of potatoes were produced in the republic during 2022, and about 3.74 thousand tons in 2023, and 92.0% of the total potatoes were grown by the farming community. In addition, 478,300 tons of potatoes were imported from 16 other countries to meet the population needs

(Jovlieva *et al.,* 2024). Among the crops, potato is one of the most important food crops, rich in minerals, and starch, and it ranks seventh in terms of the main food production in the world. For the soil-climatic conditions of Uzbekistan, potato varieties are required to meet the requirements of quick ripening, productivity, and suitability for two harvests in one year. The biochemical composition of potatoes, for example, dry matter, starch, sugar content, and other parameters determining the quality of the product, mainly vitamins, and especially their concentration, are required to be very high (Lex. uz, 2021). Therefore, the development of productive cultivars of potatoes in Uzbekistan, the identification of various diseases and the reduction of their damage to productivity are of dire need and greater practical importance. As in other crop plants, many pathogens infect the potato plant, including fungi, bacteria, mycoplasmas, viruses, viroids, and nematodes, which lead to reduced yield and quality. Among them, along with phytophthora, viruses are the most important pathogens (Ahmad *et al.,* 2012; Adolf *et al.,* 2020; Matniyazova *et al.,* 2022).

The potato crop is severely affected by various biotic stresses among which viruses play a significant contribution in terms of huge loss in crop yield worldwide including Uzbekistan. Potato is affected by deadly viruses especially more potential infection via seed tubers due to the vegetative reproduction of the crop. More than 40 viruses and viroids hamper the cultivation of potatoes across the globe (Palukaitis, 2012; Wang *et al.,* 2011). The crop is infected by more than 30 RNA viruses, out of which 13 are mainly transmitted by aphids. Potato leaf roll virus (PLRV), which belongs to the genus Polerovirus and family Luteoviridae, is a widely spread potato virus worldwide and responsible for more than 20 million tonnes of yield loss (up to 90%) globally (Kreuze *et al.,* 2020). PLRV is the only transmitted by aphids, namely, Myzus persicae. It is widely multiplied in the phloem tissue and the symptoms of the disease reflect this position (Kumar et al., 2017). Because potato is a vegetatively propagated crop, once they get infected with viruses, they can easily disseminate in the progeny tubers. These viruses are found in single or most of the time as a mixed infection within the potato crops. Tubers used for planting in the next season can harbor latent viruses that subsequently reduce emergence, plant vigor, and yield. All daughter tubers produced by infected mother tubers (secondary infection) will also get infected via systemic translocation of the virus during growth (Scheidegger and Struik, 2017; Leonard and Holbrook, 1978). More than 50 viruses have been reported to infect the potato plant worldwide, among which Y, S, A, X, and L viruses are the usually common (Acosta-Leal *et al.,* 2011), and PVM is also one such virus (Adams and Antoniw, 2004). The first information about the spread of this virus was provided in 2011 in Uzbekistan (Fayziev *et al.,* 2020). PVM is one of the most widespread and genetically diverse viruses in the world (Glasa *et al.,* 2019; Halabi *et al.,* 2021) In particular, Indian researchers who researched the diversity of this virus genome, and

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 8.5 thousand pairs of nucleotides, and it affects and reduces crop productivity by 15-45% (Xu *et al.,* 2010). The virus is transmitted by mechanical inoculation and causes local and systemic spotting in the Datura metal plant, red spot in the Vigna plant, brown ring-shaped local wound in *Nicotiana debneyi*, and linear necrosis in stem and leaf veins in *Solanum rostratum*. Chenopodium album does not cause any disease symptoms (Hiruki, 1970). The virus is transmitted to the plant by a non-persistent route, i.e., by aphids, including *Myzus persicae* Sulz, and also by 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.,* 2018).

The molecular and genetic characterization of PVM has been investigated in numerous studies, and its genome consists of 8534 nucleotide sequences and contains 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 the synthesis of a 223-kDa protein, which is an enzyme that replicates the viral RNA; the next block consists of three ORFs and encodes 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).

The main viral diseases of potatoes in the territory of 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 leads to increased economic losses in potato fields (Awasthi & Verma, 2017). In recent years, effective research on viruses infecting important crop plants has been carried out in Uzbekistan (Sobirova *et al.,* 2020, 2023; Fayziev *et al.,* 2020), including determining the level of virus infection 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), as well as studies on the effects of the viruses on the physiological properties of crop plants were being conducted (Sobirova *et al.,* 2023).

To carry out measures to combat viruses, it is important to study the biological characteristics, the level of spread of the virus using sensitive methods (Clark *et al.,* 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, the most important of them is detecting the virus in buds using fast and sensitive methods, which reduces the spread of the virus and its economic damage (Malko *et al.,* 2019). Therefore, molecular identification of PVM was the main goal of the present research work. Potato L-virus causes disease symptoms such as paleness and roughness of the leaf color, as well as upward twisting of the leaf plate in the plant. The above disease symptoms are differentiated from other potato virus diseases. For the study, the PCR method was chosen for correct and accurate diagnosis of the disease and identification of the virus, and vitamin and total protein levels were checked

Materials and methods

During 2022-2023, the genotypes used in the study were Arizona, Zarzara, Galla, and Santa from the Potato collection available at the Institute of Plant Science and Plant Genetic Resources of Uzbekistan and the breeding material collected by the Molecular Biology and Bioinformatics, Chirchik State Pedagogical University, Chirchik, Uzbekistan.

Samples collection

The KMV disease symptoms were visually manifested in the potato cultivar 'Arizona, Zarzara, Galla, and Santa' field in September 2022-2023 in the Tashkent region, Uzbekistan. The samples were stored in the Thermo Fisher Scientific (AQSH) refrigerator at -20°C and brought to the laboratory. Plant leaves were excised, placed in labeled plastic bags, and stored at −80 °C to prevent viral RNA degradation. Virus source and symptoms leave samples showing symptoms of leaves rolling; chlorosis and stunting were separately collected from naturally infected potato plants grown at different localities of Uzbekistan, during February-October of the 2022-2023 growing seasons. Agricultural technology-60-150 kg of nitrogen (based on nutrients), 100-200 kg of phosphorus, 30-60 kg of potassium mineral fertilizer, and 15-20 tons of manure are applied per hectare of the land where potatoes are planted and plowed deep. Most medium-sized (50-80 g) potatoes are allocated for planting. The rows are planted at 60 cm, 25 cm between the bushes, 8-12 cm deep. The sowing rate is 2,5-4,0 tons per hectare. The average yield in intensive technology is 42-44 t/ha.

Serological screening for infecting PLRV in potato plant leaves

Indirect enzyme-linked immunoassay (ELISA) was performed for PLRV screening as described previously by Hobbs et al. (Hobbs et al., 1987) Symptomatic and control leaves were macerated in 5 ml extraction buffer containing 0.05 M phosphate-buffer saline, 0.01 M sodium diethylene carbamide at pH 7.4. The extracts were centrifuged at 10,000g for 5 min. ELISA plate was incubated overnight at 4 °C with 100 µl of supernatant. The plate was washed and blocked with skim milk powder. Two hundred microlitre of rabbit polyclonal antibodies against PLRV-CP (Promega) (diluted 1:500 in PBS) were added to each well and incubated for 3 h at 37 °C. After

washing, secondary antibody goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (Promega) diluted 1:1000 was added to the wells and incubated for 3 h at 37 °C. The plate was developed with the substrate p-nitrophenyl phosphate (p-NPP) and OD at A405 nm was measured.

RT-PCR analysis of viral RNA in potato

Based on the results obtained by indirect ELISA, total RNA was extracted from symptomatic leaves of potatoes using the RNeasy Plant Mini kit (Thermo Scientific) following the manufacturer's instructions. RT-PCR was carried out using PLRV_CP gene-specific primers, and cDNA synthesis was carried out using an RT-PCR kit (Thermo Scientific). The primers used for molecular identification of PLRV are shown in Table 1.

Table 1. PLRV CP gene-specific primers

Names of gene	Names of primer and sequences of primer	
PLRV_CP gene	FP - AGCCGGTTTATAYTTHGTTTA	RP-GTYGGTTGTGGGCTYGG

The total reaction volume of 20 µl contained an RNA template, sterile water, 10 mM dNTPs mix, and 50 µM random nanomers. Initially, the reagents were incubated at 70 °C for 10 min, later on, sterile water, 10×RT-buffer, 20 U/µl RNase inhibitor, and 20 U/µl enhanced AMV-RT were added and incubated at 25 °C for 15 min, and 45 °C for 50 min. PCR was performed keeping the total reaction volume at 50 µl. The infected samples were first tested through DASELISA by following the standard protocol. Further from the positive PLRV samples identified through DASELISA, total RNA was extracted from the mid-rib and major veins of the infected leaf using an RNA extraction kit, and the first-strand cDNA was synthesized using a cDNA synthesis kit (Fermentas) with respective primers. Finally, to confirm the PLRV infection, RT-PCR was performed using PLRV coat protein gene-specific primers. Total RNA was extracted from all possible parts of the infected plant (viz., tuber, main shoot, branched shoot, and leaf) and electrophoresis analysis of the RT-PCR product showed a single amplified fragment of 726 bp. Further, the TA cloning approach was employed to sequence all the amplified CP genes from different parts of infected plants (Kumar et al., 2017; Tamura et al., 2021).

A sequence was carried out to determine the analysis of water-soluble vitamins according to the algorithm below.

For this, it is obtained from the above-prepared plant nodules. HPLC performed water-soluble vitamin analysis using a gradient elution mode and a diode array detector (DAD). Acetonitrile and buffer solution were used as the mobile phase. Spectral data were studied and processed in the spectral range from 200 to 400 nm (Taliansky et al., 2003)

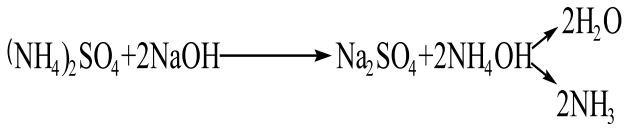
Chromatographic method conditions:

Mobile phase (gradient mode) - acetonitrile - buffer solution pH=2.92 (4%: 96%) 0-6 min., (10%: 90%) 6-9 min., (20%: 80%) 9 -15 ., (4%: 96%) 15-20 min. Injection volume - 10 µl. The speed of the mobile phase is 0.75 ml/min. Column - Eclipse XDB - C18. 5.0 microns, 4.6x250 mm. The detector is a diode-matrix detector, processes were carried out in sequence at wavelengths of 272, 292, 254, 297, and 360 nm.

The following algorithm was used to determine the amount of protein in the PVC tube: Kel'dal's method was used to determine the amount of total proteins. Based on this, the amount of total protein was calculated by determining the amount of nitrogen. The essence of the method is to hydrolyze the organic substances in the sample with the help of concentrated sulfuric acid (the amine groups in the protein) to form ammonium sulfate salts.

Nitrogen organic substances + H2SO4→(NH4)2SO4+CO2+H2O

After hydrolysis, the resulting ammonium sulfate was treated with sodium hydroxide to convert it into ammonia.



The ammonia or ammonium hydroxide formed as a result of the remaining neutralization is titrated with an acid-base solution of sulfuric acid. Nitrogen content was calculated from the calculated ammonia content. An accurate sample for analysis was taken from the average crushed homogeneous sample of the studied sample, the error rate should not exceed 0.1%. The sample was quantitatively analyzed in a Kel'dal flask. The experiment was then carried out according to the instructions (Leonard & Holbrook, 1978)

Processing of the obtained results: The mass fraction of nitrogen (X) in the analyzed sample was calculated using the formula as a percentage of the sample's mass through the volume after titration of the amount of ammonia passed through dilute sulfuric acid.

$$X = \frac{(V_1 - V_0) * K * 0.0014}{m} * 100\%$$

V_0 is the volume of the 0.1 mol/l sodium hydroxide solution, ml, used to titrate the 0.1m mol/l sulfuric acid solution increased in the sample experiment.

Results

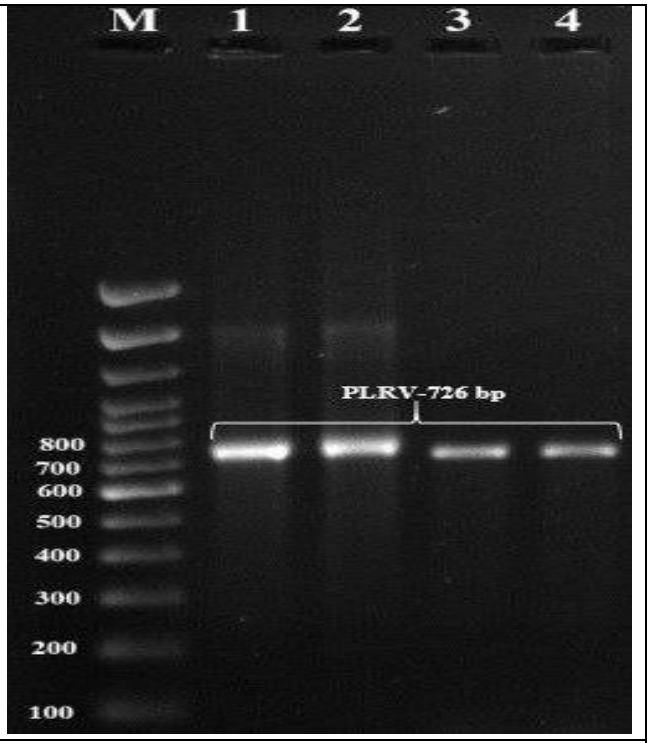
The infected samples were first tested through DAS-ELISA by following the standard protocol. Further from the positive PLRV samples identified through DAS-ELISA, total RNA was extracted from mid-rib and major veins of the infected leaf using RNA extraction kit, and first-strand cDNA was synthesized using cDNA synthesis kit (Fermentas) with respective primers. Finally, to confirm the PLRV infection, RT-PCR was performed using PLRV coat protein gene-

specific primers. Total RNA was extracted from all possible parts of the infected plant (viz., tuber, main shoot, branched shoot, and leaf) and electrophoresis analysis of the RT-PCR product showed a single amplified fragment of 726 bp (Fig. 1B).

We used the same tubers from the infected plant as a seed for next season's cropping in a controlled environment and all the results were further validated. Our consistent data from four cropping seasons indicate that these tubers act as a reservoir for these viruses and lead to the emergence of unhealthy progeny plants. From infected tubers, all progeny plant parts could get infected via systemic translocation of the PLRV. Because the aphid population increases exponentially during the late winter season, the probability that they will encounter an infected plant is higher. Aphids acquire the PLRV in the salivary glands by feeding on infected plants and further continue to transmit the virus to healthy plants for its remaining life and complete the disease cycle. Our present study also supports the findings of Leonard and Holbrook on the role of aphids in PLRV pathogenicity. Control depends solely on using disease-free seeds for planting (Scheidegger & Struik, 2017; Leonard & Holbrook, 1978). Farmers of the Indo-Gangetic Plain of this region import potato seeds from neighboring states or local companies, where the risk of getting unhealthy seeds is very high. As a result, the pressure of production of disease-free seed potatoes has become a major challenge.

diseased healthy

A



Detection of PLRV in different parts of infected potato plant using CP-specific primer(726 bp)

B

Figure 1. A-Infected PLRV Plant sample. **B**-Detection of PLRV in different parts of infected potato plant using CP-specific primer (bp).

According to the results of the conducted research, it was found that potatoes of Arizona, Zarzara, Galla, and Santa varieties are infected with KLV. It was observed that the virus infection was high in all 3 cultivars. Quantification of water-soluble vitamins from potatoes using HPLC (HPLC). Damaged and healthy tubers of the potato plant were compared (Table 2).

Table 2. Summary of Quantification of Water-Soluble Vitamins from Potato by HPLC (mg/g).

Sample name	B1	B6	B9	PP	C	B2	B12
Arizona healthy	0,045	1,421	-	-	2,572	-	-
Arizona diseased	-	0,477	-	-	1,521	-	-
Zarzara healthy	0,023	1,243	-	-	3,044	-	-
Zarzara diseased	-	0,564	-	-	2,512	-	-
Galla healthy	0.027	1,337	-	-	2,545	-	-
Galla diseased	-	0,427	-	-	1,457	-	-
Santa healthy	0,035	1,256	-	-	3,048	-	-
Santa diseased	-	0,521	-	-	2,415	-	-

In the experiment, when 4 potato varieties were infected with the virus, vitamin B1 was detected only in healthy plants. In our healthy Arizona plant, we detected B_1-0.045, B_6-1.421, and C - 2.572 vitamins, and in our infected Arizona plant, we detected lower levels of B_1-undetected, B_6-0.477, C-1.521 vitamins compared to our healthy plant. B_1-0.027 B_6-1.337 and C-3.044 in our partially healthy Galla potato variety were detected, and B1-undetermined, B6-0.564, and C-2.512 in our golden plant. No vitamins have been identified in the Galla potato. According to the results of the study, the total protein content in PVL nodules was found to be high and low in all 3 types of damaged and healthy nodules (Table 3).

Table 3. Summary of determination of total proteins in PVL nodule

Potato cultivars		Amount of total proteins %
Arizona	healthy	2.917
	diseased	2.914
Zarzara	healthy	4.426
	diseased	3.348
Galla	healthy	3.279
	diseased	3.259
Santa	healthy	4.328
	diseased	3.338

Arizona healthy 2.917 and diseased 2.914, Zarzara healthy 4.426 and diseased 3.348, Santa healthy 4.328 and diseased 3.338, Galla healthy 3.279 and diseased 3.259 percent of infected total protein was determined.

Discussion

Viruses are the principal threat to potato production all over the world, including in Uzbekistan (Fayziev et al., 2019; Jovlieva et al., 2023). As the availability of virus-free seed tuber is the main barrier to increased potato production in Uzbekistan, documentation of exact viruses in the seed lot as well as in the field is needed to enhance potato production. However, a few numbers of studies have been performed on molecular detection and infection status of potato viruses in Uzbekistan. Therefore, in this study, RT-PCR-based detection was performed to determine the present status of four important potato viruses in potato-growing regions of Uzbekistan. Detection results showed that the tuber samples were associated with PLRV, PVX, PVY, PVS, PVH, PAMV and PVM. PLRV, PVX, PVY, PVS, PVH, PVM, and PVA have been reported so far in Uzbekistan (Fayziev et al., 2020). In addition, detection results further demonstrated that mixed infection was very common, consistent with the earlier findings (Kerlan & Moury, 2008). Although a single infection by PVX is not so damaging, however co-infection with other viruses (PVY, PVA, and PVS) may produce vast yield losses (Eskendirova et al., 2019). Mixed infection of PLRV and PVY can generate huge economic losses by reducing tuber size and quality rather than solitary infection of PLRV or PVY (Fayziev et al., 2023). PLRV, PVX and PVY are more destructive potato viruses in Uzbekistan, responsible for an annual yield loss of 15–78% (Fayziev et al., 2023). Generally, single infections by PVA PVH PVM or PVS are symptomless and difficult to characterize, whereas various symptoms including chlorotic spot, rugosity and mottling of leaves, stunting, and premature leaf dropping may be observed during mixed infection of these viruses depending on the susceptibility of potato cultivars and virus isolates(Yardimci et al., 2015). Zhang et al. reported that co-infection of PLRV, PVX, and PVY produced 1.25% infection, and PLRV, PVX, PVY, and PVA produced 0.31% infection.

Conclusion

In this study, disease symptoms such as lightening and roughening of the leaf color and upward twisting of the leaf plate were studied by molecular identification, and it was observed that the total protein and vitamin contents were significantly lower under diseased plants. It was found out from the studied literature and conducted experiments that PLRV is mainly stored in tubers and that the stored virus affects the vitamin and total protein content of potatoes. To prevent the spread of this virus, it is very important to identify the natural reservoir plants and prevent them from growing in the fields, and to plant virus-free potato varieties.

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