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CHARACTERIZATION OF MDMV UNDER ECOLOGICAL CONDITIONS OF UZBEKISTAN

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SUMMARY

Several viruses currently found in the maize (*Zea mays* L.) fields showed the primary and most crucial one is the maize dwarf mosaic virus (MDMV) in Uzbekistan. The use of a combination of serological testing and next-generation molecular-genetic approaches helped assess the current reservoir of viruses observed in past disease outbreaks. The MDMV has gained proof to infect cereal grasses and maize. The presented results provide important strategies for the management of viral diseases in the sweet corn maize crop, as well as identifying potential future virus threats. The following study explores the distribution, symptoms, and diagnostics of MDMV. A pure homogeneous preparation of the virus immunized in the body of the experimental rabbit became the antiserum (Figure 1). In the isolated serum, determining the titer used the DID method, the AS titer was 1:16, and the amount of isolated AS was 12.5 ml. In addition, the conduct of molecular-genetic diagnosis used PCR. According to PCR results, MDMV appeared as the most common virus affecting maize crops in Uzbekistan. The PCR analysis revealed the maize sample with mosaic signs along the leaf veins and dwarfism was the causative agent of MDMV. The RT-PCR method performed relied on a fragment of the gene responsible for the synthesis of the MDMV shell.

Keywords: Maize (*Zea mays* L.), cultivars, maize dwarf mosaic virus (MDMV), phytoviruses, plant diseases, yield-related traits, productivity

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Key findings: The diagnostic results showed a considerable spread of MDMV in the maize (*Z. mays* L.) fields of Uzbekistan. The study obtained and identified a specific MDMV serum by molecular-genetic methods.

INTRODUCTION

The growing population makes it crucial to enhance the production of safe, high quality, and nutritious food to overcome hunger and malnutrition (Sattorov *et al.,* 2020). Maize (*Zea mays* L.) is one of the most widely grown crops worldwide, used for human consumption, animal feed, and as a feedstock for the production of biofuels and other byproducts (Xie *et al.,* 2021). In previous decades, maize viruses have been a highlight, especially in the context of agriculture. Viral diseases cause symptoms of varying severity, depending on the host plant type, including spotted mosaic (Fayziev *et al.,* 2023) and linear mosaic along leaf veins and ringspots (Sobirova *et al.,* 2020). For the identification of viral diseases, the development of various advanced methods has been progressing, most of which are immunological, based on the detection of a virus-specific antigen using antibodies prepared for it, as well as PCR methods based on detecting particular fragments of viral nucleic acid (Zielińska *et al*., 2012; Akhmadaliev *et al.,* 2024; Jovlieva *et al.,* 2024).

The maize dwarf mosaic virus (MDMV) is one of the most critical plant viruses with four strains (A, D, E, and F), posing a significant threat to maize production worldwide (Ludmerszki *et al.,* 2014; Wijayasekara and Akhtar, 2020; Ramazonov *et al*., 2020). In addition to this virus, several viruses existed that are equally dangerous to growing maize. These viruses are available in Table 1. Maize crops and other cereals are considerably certified hosts of the MDMV. Moreover, MDMV can cause various symptoms, such as leaf mosaic, plant dwarfing, and decline in production. The MDMV's first disclosure was in 1963 in Ohio, USA. The MDMV's transmission naturally is via aphids in a non-persistent manner, also with easy transfer by inoculation under laboratory conditions. In susceptible maize genotypes, the plants infected with MDMV showed mosaic symptoms on leaves that are visible as early as 5–7 days after inoculation (Xie *et al.,* 2021; Sobirova *et al*., 2023). MDMV overwinters in the rhizomes of sorghum (*Sorghum halepense* L.), and then its transmission to healthy plants by aphids mechanically follows. Young plants at the 5–7 leaf stage appeared more susceptible to the virus. However, at the later stages of plant development, the virus effects become weak (Davranov, 1984). Various aphid species work as virus vectors; however, the most important one is *Rhopalosiphum maidis* Fitch.

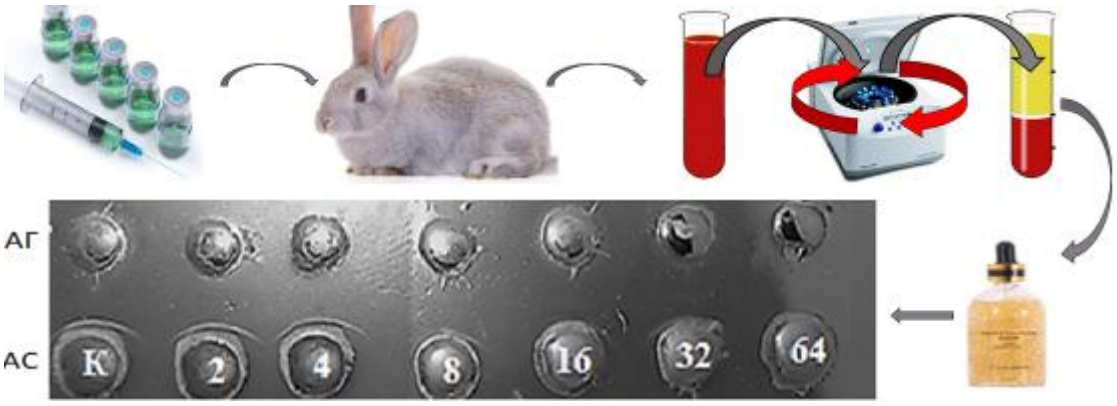

Figure 1. Preparation of antiserum and determination of its titer by the DID method.

Table 1. Viral diseases and their causative agents.

Names of viral diseases	The causative agents of viral diseases
Mosaic disease of maize	Maize mosaic virus
Lethal necrosis of maize	Viral complex: Maize Chlorotic striatum virus, Maize mosaic virus, wheat mosaics-streaked virus
Mosaic Johnsongrass	Johnsongrass mosaic virus
Chlorotic dwarfism of maize	Maize virus chlorotic dwarfism virus
Chlorotic mottling of maize	Maize chlorotic crackworm virus
Dwarf maize mosaic	Maize dwarf mosaic virus (MDMV) A, D, E, and F
Maize line	Maize line virus
Striation (streaking) of maize	Maize striation (streaking) virus
Red streakiness in maize	Wheat mozaic virus
Мозаика Сорго	Sorgo mozaic virus
Sugarcane Mosaic	Sugarcane Mosaic virus

For diagnosis of MDMV, various laboratory methods can be applicable, such as mechanical inoculation of healthy plants, immunological methods, ELISA (enzyme-linked immunosorbent assay), electron microscopy, and PCR (Tosic *et al*., 1990; Kannan *et al.,* 2018). Molecular diagnostic methods provide faster and more sensitive resolution, as well as accurate and rapid taxonomic identification of pathogens (Sherimbetov *et al.,* 2020). The taxonomic study of Maize dwarf mosaic virus (MDMV) as an important pathogen of maize, sorghum, and sugarcane has attracted worldwide attention. Considerably, MDMV has become familiar as belonging to a strain of sugarcane mosaic virus (SCMV) (Gell *et al*., 2010; Gell, 2011). The MDMV also belongs to the subgroup of sugar mosaic viruses (SCMV). Based on the identification of the parental RNA sequences, two types of RNA recombination events can be distinctive. In the case of homologous recombination, either the recombination is accurate due to high sequence similarity or inaccurate (aberrant), resulting in minor variations, such as mutations, insertions, and deletions in the molecules. In the non-homologous recombination case, the recombination event occurs between two related RNA molecules (Giolitti *et al*., 2005; Gyöngyvér *et al.,* 2014; Kanwal *et al.,* 2024). This issue also requires proof at the molecular-genetic level. Currently, these features serve as the basis for practical identification (Mohammadi *et al*., 2006).

The MDMV, like other potyviruses, is a positive-strand RNA virus (McDaniel and Gordon, 1989; Cao *et al.*, 2011; Achon *et al.*, 2012; Sobirova *et al.*, 2023). The genome comprised 9,500 nucleotides, with a covalently linked viral-genome-associated protein, VPg, at the 5′ end and a poly(A) tail attached to the 3′ end (Gell *et al*., 2010). The capsid protein (CP) (Marie-Jeanne *et al*., 2000) was the last cistron encoding conserved among many potyvirus species (Marie-Jeanne *et al.,* 2000). As per the amino acid analysis, the molecular weight of this protein coat of MDMV was 28.5 × 103 (Hill *et al*., 1973). The C-terminal end of the coat protein plays a vital role in encapsidation and cell-to-cell transmission, and the flexible N-terminus participates in long-distance and systemic transport, with the essential transport of aphids (Petrik *et al.,* 2010; Kannan *et al.,* 2018).

The MDMV genome, when translated into a single 338 kDa polyprotein, is later proteolytically cleaved into 10 different protein products that also contain a conserved short protein, known as the Potyviridae rather intriguing ORF (PIPO) in cistron P3 (Lübberstedt *et al*., 2006; Wijayasekara and Akhtar, 2020; Yeginbay *et al*., 2024). MDMV transmission can easily proceed mechanically by juice inoculation. The inactivation temperature of MDMV was within 50 °C–55 °C, the limiting dilution was 1:1000, and the survival time in juice at room temperature was

about 22–24 hours (Kannan *et al.,* 2018). The virus reached degradation at a pH value close to 9.0. When the juice acidified to pH 5.0-4.5, no loss of infectivity occurred; however, at a pH level of 4.0, the infectivity was lost. Once the MDMV presence emerges in the plant, it is crucial to implement control strategies to reduce further yield losses.

Arthropods play an influential role in transmitting all the economically important viral diseases, including MDMV disease in maize (Kannan *et al.,* 2018). Intensive control of the virus requires the use of complex methods, i.e., eliminating weeds and virus carriers. For the prevention of viral diseases, it is vital to select virus-resistant maize cultivars. Past studies revealed different maize cultivars have varied responses in resistance to viral diseases (Sobirova *et al*., 2020; Makhmudov *et al*., 2023). Based on the foregoing, the presented study aimed to assess the current reservoir of viruses' observed in past disease outbreaks through the combined use of serological testing and next-generation molecular-genetic approaches.

MATERIALS AND METHODS

Immunological method for MDMV diagnostics

The immunological method centered on the detection of the virus and its antigens using specific antibodies. The virus identification followed a modified method (Ouchterlony*,* 1953). In this case, 1% Difco agar as dissolved in 0.1 M phosphate buffer (pH 8.0), received 24 ml of liquid agar cooled to 60 °C–65 °C and poured onto a glass plate measuring 9 cm × 12 cm with a 'line' (water scale) and placed horizontally. After the agar-agar gel had solidified, the wells' preparation in a straight line utilized special stamps with a distance of 4 mm between the holes. After adding 80 µl of AG and AC to these wells, the placing of glass plates in a special device followed, and their keeping in a desiccator with 200 ml of water at the bottom remained for 48 h. During this period, the diffusion of AG and AT transpired, forming the precipitation lines from their specific collision (Fayziev *et al*., 2020). Sections of agar with the results of each reaction attained placement on a defatted glass slide to dry for 15–20 h at 20 °C, covered with filter paper. Then, soaking it under water ensued, followed by cleaning with filter paper. The purified agar pieces' transfer in a special dye consisted of a mixture of 1% amido black methanol, acetic acid, and water (4:4:1) for 10 min to stain the precipitation lines, after which washing the preparation several times employed a 'washing liquid' (methanol, acetic acid, and water) (Fayziev *et al.,* 2020). After drying, reading the reaction results continued.

For the study using DID, plant sample preparations were as follows: a plant leaf crushed in a mortar had the juice isolated and mixed with 0.01 M phosphate buffer (pH 7.5) in a ratio of 1:1, then incurred centrifugation at 8000 rpm for 10 min. After centrifugation, the collected supernatant served as the AG.

Virus diagnostics by PCR method

For the determination of the species composition of viruses affecting maize, the study used the molecular-genetic method PCR. Polymerase chain reaction is a method of molecular biology that allows achieving a higher enhancement in low concentrations of certain fragments of nucleic acid (DNA) in a biological sample. Samples collected from maize fields underwent diagnosis using the RT-PCR method.

The use of the Gene JET Plant Genomic DNA Purification Mini Kit (Thermo Scientific) reagent helped detect the nucleic acids. PCR has many varied types, considering the purpose of the work and the components of the object. If the object has an RNA genome, then applying PCR with reverse transcriptase (RT-PCR) was effective. The RT-PCR used aided in detecting the RNA molecules in a sample with a previously known sequence region complementary to the primers. Exponential amplification using RT-PCR is a sensitive technique that can detect small amounts of RNA molecules. The RT-PCR process had several stages: design and production of virus-specific primers, extraction of genomic RNA, production of cDNA, and

amplification of viral DNA. In conducting RT-PCR for viruses affecting maize, several primers were selected alternatives from the scientific literature.

Extraction of the viral genomic RNA

In virus-infected samples, the isolation of viral genomic RNA proceeded using the Purelink RNA minikit from Invitrogen (ThermoFisher, USA) as per attached instructions. For this purpose, samples of maize plants with symptoms of MDMV disease succeeded collection and extraction by placing 5 g in a porcelain mortar and adding liquid nitrogen. The 200 µl of the prepared extract, upon removal, attained placement in a 2-ml Eppendorf tube before being thoroughly mixed on a vortex (Vortex MX-S, DLAB), after which, mixing 1.5 ml of lysis buffer (prepared in advance by adding 10 µl of 2-mercaptoethanol per 1 ml of lysis buffer) ensued. Then, its incubation for 3 min at room temperature and centrifugation for 5 min (2600 rpm) took place. The supernatant, as separated, succeeded in transferring in a new tube. The 96% ethanol when added had a ratio of 1:1.5 to the volume of the supernatant and continued mixing by vortex. Its pouring into a tube with a filter included in the kit sustained centrifugation for 15 s (12,000 rpm), before separating the sediment. The 700 µl of wash buffer (wash buffer I) after adding to the sediment incurred centrifugation again for 15 s (12,000 rpm), with the lower part of the column tube discarded and replaced with a new tube. Then, adding the second wash buffer (wash buffer II) in a set of 500 µl underwent centrifugation for 15 s (12,000 rpm). Removing the filter part of the tube proceeded its placement in a new sterile tube, with 100 µl of sterile RNase-free water slowly added from the center of the tube and incubated at room temperature for 2 min. During this time, its centrifugation (for 2 min at 12,000 rpm) continued to isolation of the filter portion of the tube.

Obtaining c-DNA by reverse transcription

In obtaining the cDNA based on the genomic RNA of the viral matrix or total reverse transcription (TT) RNA, the experiment used the following reagents and materials: dN6 (Evrogen, Russia), oligo-dT20 primer (specific to the poly-A sequence at the 3' end of the viral RNA) or reverse (antisense) primer (Akhmadaliev *et al*., 2024). Several primers used in the diagnosis of viruses that infect corn included the VKMK primers:
F - 5' - CAACCAGGGCYGAATTTGATAG -3' end,
R – 5 '- GTGCAAGGCTRAAGTCGGTTA - 3' end (Kannan *et al.,* 2018). The 2 µl (20 nmol) of oligonucleotide primer and 5 µl of Triton eluate or total RNA succeeded placement in a thin-walled microcentrifuge tube (SnapSeal Graduated Microtubes, SSIbio, USA).

The mixture remained in a thermostat at 65 °C for 5 min to bind the primers. After incubation, the tubes remained in a container with ice. After vapors appeared on the walls of the tube, centrifugation followed until the liquid drained to the bottom of the tube, afterward, adding the TT mixture. Preparing the TT mixture (1 reaction) will require the following: 4.5 µl water (nuclease-free water, Thermo Scientific), 4 µl 5x MMLV reverse transcriptase buffer (Eurogen), 2 µl 10 mM deoxynucleotide triphosphate (dNTP) mixture, 2 µl dithiothreitol (Eurogen), ribonuclease inhibitor (0.5 µl RiboLock, 40 units/µl, Thermo Scientific), and 1 µl MDMV reverse transcriptase (Eurogen).

The use of reverse transcriptase enzymes also materialized, as obtained from other companies: Im Prom II (Promega, USA) and Super Script II (Invitrogen, USA). The reaction took place at 42 °C in a Termit thermostat for 60 min. After the reaction's completion, the tubes kept in a thermostat at 70 °C remained for 10 min to inactivate the enzyme before their placement in a container with ice. Then the PCR proceeded in an amplifier (TOUCH.T 960 amplifier). By carrying out the said process, the PCR product detection method employed 'by endpoint' (electrophoretic). The calculation of the optimal annealing temperature of the primers used the gradient PCR method:
1. 94 °C - 2 min; 2. 94 °C - 30 s; 3. Gradient 54 °C - 30 s; 4. 72 °C - 45 s; repeating steps 2–4 45 times; and final step 5. 72 °C - 5 min.

Gel electrophoresis provided a complete picture of the PCR product. The

method continued in 2% agarose gel, using ethidium bromide as an intercalator, with the voltage at 80–110 V for 80 min. The gel fixing used the BioDocAnalyze system (Biometra).

RESULTS

MDMV diagnostics by molecular-genetic method

The preparation of virus-specific antiserum included several stages. For this purpose, injecting a pure homogeneous preparation of MDMV into the femoral muscles of a rabbit and under the skin of the scapula at a concentration of 4 mg/ml proceeded five times. For each injection, adding 1 ml of Freund's adjuvant occurred to 1 ml of the viral preparation and mixed well until forming a stable emulsion, since thorough mixing can introduce air into the mixture and cause discomfort in the rabbit's body. Fourteen days after the last immunization, 10 ml of blood taken from the ear vein of the rabbit ensued and another 10 ml three days later.

After keeping the collected blood at room temperature for one day, the blood serum incurred slow draining to acquire the supernatant, which acquired centrifugation at 2000 rpm for 5 min to remove the remaining formed elements. Determining the AS titer employed the DID method. The antiserum, which determined the titer, remained in separate 0.6 ml AS vials, adding 1-2 drops of chloroform before its storage at +4 °C. The isolated AS amount was 5 ml during the first blood draw and 7.5 ml during the second blood draw; from a total of 20 ml of blood drawn, the AS was 12.5 ml. If using 10 µl (per small well) of AS will test one sample with the DID method, 100 µl is for 10 samples, and 1 ml is for testing 100 samples, the 12.5 ml of undiluted AS will reach 1250 samples. However, the above experiments' determination needs AS diluted at 1:16. Thus, 12.5 as diluted at 1:16, which was 100 ml, will mean 12.5 ml is enough to test 1250 samples, and 100 ml = 10,000 samples. The resulting antiserum served in immunodiagnostics of MDMV and was of great economic importance. Thus, a specific antiserum to MDMV succeeded in its preparation. The titer of AS as determined by the DID method at 1:16 obtained the AS used to diagnose the MDMV.

MDMV diagnostics by the RT-PCR

Several primers were applicable for the RT-PCR; however, the obtained MDMV primers came from the following:
F - 5 '- CAACCAGGGCYGAATTTGATAG -3',
R - 5 '- GTGCAAGGCTRAAGTCGGTTA - 3' (Kannan *et al*., 2018).

Ordering and receiving specific primers occurred from the DT Technology Belgium. These primers showed positive results, and the results of the PCR product appear in Figure 2. For the isolation and synthesis of nucleic acids, using a set of reagents (Gene JET Plant Genomic DNA Purification Mini Kit, Thermo Scientific) also took place. After obtaining the cDNA, a further amplification process proceeded as a classical PCR, carried out in a TOUCH.T 960 amplifier (Table 2).

Electrophoretic detection of PCR product, known as 'endpoint,' helped analyze all the samples. Gel electrophoresis, as performed in 2% agarose gel, used ethidium bromide as an intercalator at 110 V for 80 min. Then, fixing the gel engaged the BioDocAnalyze system from Biometra. According to the literature, the MDMV gene responsible for the capsid protein (CP) has 814 nucleotide pairs; however, for diagnostics, we used several primers. Primers responsible for the gene fragment counted 336 bp and provided positive results.

The figure showing the PCR product providing positive results means that the most common virus affecting the maize was MDMV. The result of the PCR analysis established that the sample isolated from the corn plant with symptoms of yellow mosaic along the veins of the leaf and dwarfism is the causative agent of MDMV, which further gained confirmation by the PCR diagnostics method. Thus, based on the results, plants with striped yellow mosaic along the veins and signs of dwarfism in maize can attain molecular identification as MDMV. This data will reach submission to the

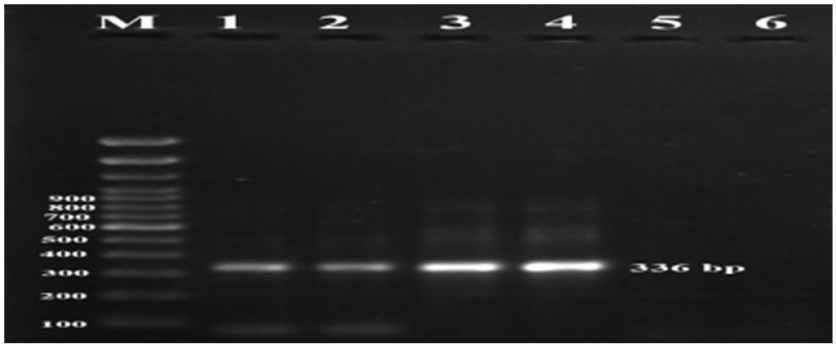

Figure 2. Photograph of gel electrophoresis of PCR ducts.
M - O'GeneRuler 1 kb DNA ladder (Fermentas) and 100 bp DNA ladder Plus; 1, 2 - probe isolated from the sorghum (*Sorghum halepense* [L.] Pers); 3, 4 - probe isolated from a maize plant with symptoms of streak mosaic along leaf veins and dwarfism (Figure 1, g), 5, 6 - probes obtained from healthy maize samples. Primers MDMVF/MDMVR - obtained from the article by Kannan *et al*. (2018) and used for molecular identification of MDMV virus.

Table 2. Stages of the PCR.

Stage name	Temperature (°C)	Time	Duration
Primary denaturation	94	2 min	1 cycle
Denaturation	94	30 s	45 cycles
Annealing	54	30 s	
Elongation	72	45 s	
	72	5 min	1 cycle

'Collection of unique objects of phytopathogenic and other microorganisms' at the Institute of Experimental Plant Biology and Genetics at the Academy of Sciences of Uzbekistan.

DISCUSSION

The Poaceae family cultivated plants often sustain exposure to viral diseases. This is because cereals, including important major crops such as maize, wheat, barley, and oats, are prone to various viruses, such as BYDV and MDMV, which are widespread and affect the cultivated and wild species of the Poaceae family. The virus is also common on Johnson grass plants in the border zones of maize fields, and it seems the origin of the maize virus is from Johnson grass, and the virus is a special strain of sugarcane mosaic virus, which also infects Johnson grass (Mohammadi *et al.,* 2006). MDMV causes mosaic on leaves and dwarfing of plants with reduced production. Noticeable symptoms include yellow and light green spots and streaks on leaves, as well as general stunting of plants. The virus infection rate was 47% in 2020; however, after the application of the newly developed control measures, the infection rate declined to 23% in 2023.

Among the various virus diagnostic methods used, the molecular-genetic method differed from other methods in its sensitivity (Boonham *et al.,* 2014). The diagnosis relied mainly on the CP gene, providing effective results and allowing the detection of mutation that occurs in the viruses (Gell *et al.,* 2010). Immunological methods are often applicable in the virus biology studies nowadays. The development of these methods requires the preparation of specific serum against viruses, and therefore, the immunoenzyme analysis can be successful for its production (Ouchterlony, 1953; Akhmadaliev *et al*., 2024).

The highest infection rate (47%) was notable in 2020, and agronomists faced the challenge of developing effective control

measures against the virus. However, through the implementation of integrated management strategies, including the use of resistant varieties, optimized agronomic practices, and biological control methods, the situation improved significantly by 2023. The infection rate has decreased to 23%, indicating the positive impact of the measures taken. In parallel, the farming community has started to emphasize regular monitoring for early detection of infections and rapid response. The undesirable consequences caused by MDMV require farmers to be constantly vigilant and innovative in their crop management approaches. The integrated approaches will also minimize losses and increase the crop's resistance to viral infections in maize crops in the future. Over the past 80 years, great strides have been successful in the understanding of plant immunity to viruses. Although most of the known natural resistance genes have long had domination by R genes (encoding NBS-LRR proteins), numerous recessive crop resistance genes have gained cloning over the past decade, highlighting another evolutionary strategy for blocking viruses (Nicaise, 2014).

The evidence-based symptomatic and biological studies carried out by identifying viruses using serological and molecular-genetic methods increased the level of reliability of the results (Ludmerszki *et al.,* 2014; Akhmadaliev *et al.,* 2024). These diagnostic methods confirmed the presence of infection and controlled the spread of the virus, monitored the resistance of different cultivars, and took measures to manage crop production to minimize damage from the viral diseases. Modern molecular technologies, such as PCR, DNA, and RNA sequencing, and hybridization techniques, lead to rapid and accurate detection of viral infections. It is also vital to apply molecular diagnostic techniques at the early detection stage of viruses, which greatly facilitates measures to control and prevent their further spread.

The introduction of modern technologies into practice will enable phytopathologists to manage already known viruses, as well as predict the emergence of new viral threats. To date, our research has identified virus diseases of maize fields' symptoms, done monitoring of spread, diagnosed and established the virus species by molecular identification, and established the damage of the virus to the quantity and quality of the crop.

CONCLUSIONS

The specific antiserum to MDMV was successful in its isolation and preparation, with the obtained antiserum used for MDMV diagnosis. The PCR analysis confirmed that in maize samples, the symptoms of yellow mosaic on leaf veins and dwarfism appeared to be the causative agent of MDMV. Thus, MDMV's isolation and transfer to the 'collection of unique objects of phytopathogenic and other microorganisms' at the Institute of Experimental Plant Biology and Genetics at the Academy of Sciences of Uzbekistan achieved submission successfully.

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