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Molecular Genetic Identification of Cucurbit Aphid-Borne Yellows Virus

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Abstract The article examines the symptoms of luteovirus infection in chickpea fields cultivated in various ecological regions and analyzes the prevalence of the disease using the TBIA method. Additionally, the cucurbit aphid-borne yellows virus, belonging to the Polerovirus genus, was identified as being widespread in chickpea fields.

Keywords Luteovirus, TBIA, Yellows, Stunting, Chloros, RT-PCR, Luteoviridae

1. Introduction

The chickpea plant is grown in arid regions of the Republic of Uzbekistan, including the Jizzakh and Tashkent regions and some districts of the Kashkadarya region. In agricultural production of biologically pure food products, biotic factors such as phytopathogenic microorganisms, bacteria, fungi, viruses, and abiotic factors cause significant economic damage by drastically reducing productivity. It has been established that chickpea plants worldwide are naturally infected by 40 viruses, belonging to 19 genera and 11 families [1,2]. In most countries, viruses such as the *bean leaf roll virus (BLRV), soybean dwarf virus (SbDV), chickpea chlorotic dwarf virus (CpCSV), chickpea chlorotic dwarf virus (CpCDV),* and *beet western yellows virus(BWYV)* affect chickpea plants and cause up to 30% economic damage [2-4]. Although leaf yellowing is observed with the viruses as mentioned above, the initial symptoms of chickpea infection differ in certain morphopathological features. Specifically, the BLRV, CpCSV, and CpCDV viruses are characterized by distinctive symptoms such as leaf curling and stunting [2-4]. To monitor the spread of luteoviruses in various ecological regions of our republic, phytovirological analyses were conducted using ELISA and PCR methods in chickpea fields in the Kashkadarya, Jizzakh, and Tashkent regions. In the chickpea fields where phenological observations were carried out, symptoms of luteovirus diseases were identified. Biological samples from chickpea plants exhibiting disease symptoms were collected for ELISA and PCR analysis.

2. Material and Methods

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Immunochromatographic (TBIA) and molecular-genetic methods were used in the phytovirological analyses conducted in various ecological regions.

Immunoblotting Method with Nitrocellulose Membrane:

Buffers used in TBIA

1. PBS (pH-7.4) phosphate buffer saline

8.0 g (NaCl)	Make up 1 liter H_2O
0.24 g (KH_2PO_4)	
1.44 g (Na_2HPO_4)	
0.2 g (KCl)	
0.2 g (NaN_3)	

2. PBS-tween (PBST)

PBS+0.5 ml (Tween 20) per liter

3. Conjugate buffer

PBST+2% PVP (Sigma PVP-40 Polyvinyl pyrrolidone +0.2% EGG Albumin (Sigma A-5253)

4. Preparation of substrate solution:

Tube 1: p-Nitro Blue Tetrazolium (NBT) (Nitrotetrozolium Blue chloride, Sigma-Aldrich, N6876) a stock solution of 25 mg/ml in 70% diemethyformamide. Store -20℃.

Tube 2: 5-Bromo-4-chloro-3-Indolyl phosphate (BCIP) (Sigam-Aldrich, B8503) a stock solution of 25 mg/ml in undulited diemethylformamide. Store -20℃.

Substrate buffer: 0.1 M tris, pH 9.5 containing 0.1 M NaCl+5mM $MgCl_2$.

Then add 20 µl NBT solution (Tube 1) and 20 µl BCIP solution (Tube 2) to 5 ml substrate buffer and gently mix. The substrate solution should be freshly prepared just before use [5-7].

Polymerase Chain Reaction (PCR) Method: Forty chickpea plants with typical symptom of luteoviruses including leaf yellowing with interveinal chlorosis, reddening of the

flag leaf, stunting, delayed maturity were collected from chickpea growing fields of the Tashkent region in April-May 2022. The samples were transported to the laboratory, the plants' leaves were cut off, placed in plastic bags with labels, and kept at -80℃ to prevent viral RNA degradation.

Extraction and purification of total RNA from infected wheat: Total RNA was extracted from the symptomatic leaves using Mackenzie RNA Extraction Protocol (Using QIAGEN Rneasy extraction kit Cat No./ID: 74104 Germany). Leaf samples (0.1 g) were ground in liquid nitrogen, homogenized in the lysis buffer with the addition of mercaptoethanol and incubated for 3 min at room temperature. The tubes were centrifuged at 5000 r min for 5 min, and the supernatant was transferred to a new centrifuge tube. The next stages (RNA precipitation using ethanol, placing the sample on a spin-column, washing the columns with RW1 and RPE buffers and elution with elution buffer) were carried out according to the manufacture's protocol. Measurement of quantity and quality of total RNA were performed using a spectrophotometer NanoDrop Eight (Thermo Fisher Scientific, USA), then the RNA samples were stored at –80℃ until used for RT-PCR.

Preparation of cDNA by reverse transcription: For obtaining cDNA based on viral matrix genomic RNA: Total RNA was extracted from 3 samples collected from chickpea fields of Kashkadarya, Jizzakh, and Tashkent regions and cDNA synthesis was carried out in 2 stages. The primers listed in (Table 1) were used to synthesize cDNA from RNA.

Table 1

Primer name	Sequence (5´-3´)	G+C (%) content	PCR product size (bp)
Pol3870-F	ATCACBTTCGGGCCGWSTYTWTCAGA	62°	370 bp
AS3-R	CACGCGTCIACCTATTTIGGRTTITG	60°	

Step 1: Total RNA 3 µl, ddH2O 1 µl, dNTP (10 mM) 1 µl, reverse primer 0.5 µl. The mixture is heated in the PCR amplifier at 65℃ for 5 minutes and quickly removed on ice.

Step 2: 5X first standard buffer 2 µl, DTT (0.1M) 1 µl, ddH2O 0.5 µl, M-MLV RT 0.5 µl were added to the mixture prepared in the first step, and it was performed in the order shown in the PCR amplifier (Table 2).

Table 2

37℃	37℃	70℃
2 minutes	50 minutes	15 minutes
1 sycle		

Amplification of CABYV by PCR: Amplification of CABYV was conducted by polymerase chain reaction (PCR). For this, 4 µl of the cDNA was taken and PCR-mixture (for 1 reaction) 5.9 ddH_2O, 2 µl of 5X Master Mix, 0.5 µl of AS3 1 primer (table 1), 0.5 µl of Pol3870 primer were added. PCR was performed on the T960 PCR Thermal Cycler (China). The following thermocycling program was used for PCR: initial denaturation 95℃ for 1 min, then 35 cycles of (95℃ for 30 sec, 62℃ for 20 sec, 56℃ for 10 sec, 72℃ for 30 sec) followed by a final extension of 72℃ for 3 min, and 15℃ (pause).

Table 3

Name of stage	Temperature	Time	Duration
Basic denaturation	95℃	1 minut	1 sycle
Denaturation	95℃	30 sec	35 sycle
Location of the primer to the DNA (annealing)	62℃	20 sec	
Elongation	56℃	10 sec	
Finally elongation	72℃	30 sec	30 sec

Analysis of PCR products was determined by electrophoresis on a 1.5% agarose gel prepared in 10x tris-borate-EDTA buffer (TBE, Thermo Scientific), Red safe™ (INTRON Biotechnology Korea). 5 µl of dye (6x DNA loading de, Fermentas) is added to 10 µl of the sample and placed together with the sample in the agarose well. A mixture of 100 bp DNA Ladder (Invitrogen, USA) molecules of known DNA size was used as markers. Electrophoresis was performed using a horizontal Electrophoresis Mini-Sub cell GT (BIO-RAD, USA) at 100 V for 60 min. The gel was analyzed and photographed on a Gel Doc XR+ (BIO-RAD, USA).

3. Research Results

Figure 1. Symptoms of luteovirus disease in chickpea plants (A, B) yellowing of the leaves in the third layer C) yellowing of leaves from the edges and, D) the leaves roll and turn red

Results of the research: Phytovirological analyses were conducted in the fields of Iran, Halima, Gulistan, Yulduz, Jizzakh region, Jakhongir, Malhotra, and the Tashkent region of CIEN-W-19 Chickpea International Elite Nursery for Winter varieties of chickpea in the Kashkadarya region. The monitored chickpea fields detected disease symptoms

such as yellowing, reddening, twisting, and smallness of leaves characteristic of luteoviruses (Fig. 1 A, B, C, D). The yellowing of the leaves was observed to be more widespread than other disease symptoms.

The TBIA method was used to quickly and accurately determine the spread of luteoviruses in chickpea fields [5-7]. During the conducted scientific research, 30 chickpea plants in the Kashkadarya region, 28 in the Jizzakh region, and 44 in the Tashkent region were cut by cutting 10-15 cm of the stem of chickpea plants with scissors. A total of 102 biological samples were collected by placing the chickpea plants with disease symptoms in polyethylene bags. Chickpea plants collected in the field were subjected to immunoblotting in the laboratory on a nitrocellulose membrane in the prescribed manner (Fig. 2 B). Immunoblotting biological samples were wrapped in dry paper and stored in a -20° refrigerator for PCR analysis.

Figure 2. a) yellowing of leaves b) sample blotting membrane c) membrane antibody treatment process d) luteovirus detection membrane samples (400x)

Infected chickpea plant samples were treated with the membrane in the order described in the working methods section. Highly qualified scientific staff of the Virology Laboratory of the ICARDA International Scientific Research Center located in the city of Beirut, Lebanon. by Safaa Kumari, and Abdelrahman Moukahellar. In this case, the TBIA , on which the samples were first printed, was washed 3 times with PBST [pH-7.4] buffer, and 15 ml of conjugate buffer PVA was added to the TBIA and left on a shaker for 1 hour. Conjugate buffer-treated TBIA was washed 3 times with PBST buffer. In the next step, the specific 5G4 monoclonal antibody, which binds to the protein receptors (epitopes) in the shell of the chickpea-causing Luteovirus virion, was diluted 1:500 in 15 ml of PBC buffer, placed on the TBIA and left on a shaker at room temperature for 1 hour. After treatment with broad-spectrum monoclonal antibody 5G4, the membrane was washed 3 times with PBST, 15 ml of conjugate buffer was added to TBIA and 1500 µl of NBT and PBIP were added to stop the reaction and left for 5 min. To study the results of the reaction, TBIA was analyzed under a light microscope [x400] (Figure 2 D).

When TBIA was viewed under a microscope, 60% of samples were found to be infected with Luteoviruses (Figure 2 D). Monoclonal antibody 5G4 is broad-spectrum (universal) and its sensitive part has the property of recognizing all luteoviruses infecting chickpea plants with high accuracy, but it cannot be used for serological identification of viruses. In the detection of phytoviruses by immunological method, the use of monoclonal antibodies obtained for viruses is required.

The scope of scientific research was expanded and PCR analyses were carried out by extracting total RNA from plants from biological samples in which luteovirus infection was detected in the order indicated in the working methods section.

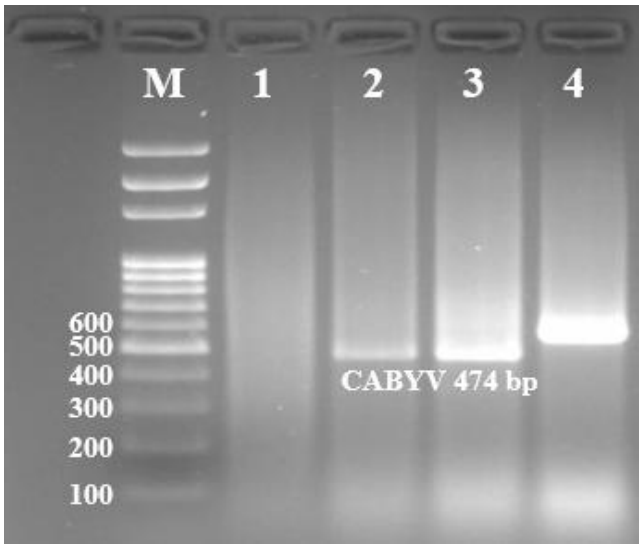

Figure 3. Cucurbit aphid-borne yellows virus molecular-genetic identification, 1-3 chickpea plant samples 4 positive control

In the course of scientific research, it was found that luteoviruses were spread in the chickpea fields of the Jizzakh and Tashkent regions, where photobiological analyses were carried out. CABYV was amplified at 474 bp for the first time in Uzbekistan according to the results of PCR analysis in the samples collected in Tashkent and Jizzakh regions where luteovirus infection was detected by the TBIA method (Fig. 3). Taking into account the complex damage caused by luteoviruses to plants, the goal of molecular-genetic identification of viruses in samples with luteoviruses was determined.

4. Conclusions

The main host of the Cucurbit aphid-borne yellows virus, which belongs to the Luteoviridae genus of poleroviruses, is the pumpkin plant. In the conditions of Uzbekistan, CABYV was detected for the first time in a pea plant. Yellowing of leaves typical of luteoviruses was observed in pea plants infected with CABYV. It was also found that it is widespread in the Tashkent region.

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