Journal of Plant Biochemistry & Physiology

Asad et al., J Plant Biochem Physiol 2019, 7:1
DOI: 10.4172/2329-9029.1000231

Research article Open Access

Serological and Molecular Identification Based on Coat Protein (CP) Gene of *Cucumber mosaic virus* (CMV) Infecting Cucumber (*Cucumis sativus* L) in Pothwar Region of Pakistan

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Received date: February 26, 2019; **Accepted date:** March 13, 2019; **Published date**: March 20, 2019

Abstract

Cucumber mosaic virus (CMV) is one of the most important plant viruses and a major threat to a wide range of hosts. Prevalence of CMV in Pakistan is alarming for vegetable production especially cucurbits. The present study was done to estimate the prevalence, distribution as well as coat protein base identification of this notorious virus. During 2015-16 incidence of CMV was recorded in cucumber field in the Pothwar region of Pakistan (Rawalpindi, Attock, Jhelum, Chakwal, and Islamabad). During survey 150 samples were collected and tested through DAS-ELISA (Double Antibody Sandwiched Enzyme Linked Immunosorbent Assay). Results show that CMV prevails throughout the region. Maximum disease incidence was recorded in Rawalpindi (50%) followed by Chakwal (46%), Attock (43%), Islamabad (40%) and Jhelum (36%). Virus infectivity was assayed by indicator plants (*Capsicum annuum, Cucumis sativus cv, Chenopodium amaranticolor, C. quinoa, Nicotiana tabacum, and Datura stramonium*) through mechanical inoculation. Upon mechanical inoculation, plants show Chlorotic lesion, Necrotic lesion, Mosaic, Stunting, Spots. Coat protein (CP) gene-specific forward (CMVF-45) and reverse (CMVR-45) primer amplified 500bp fragments through Polymerase Chain Reaction (PCR).

Keywords: CMV; ELISA; PCR; Coat protein (CP)

Introduction

The Pothwar region is situated in the north-east part of Pakistan and encompasses four districts (Rawalpindi, Jhelum, Chakwal, Attock) and Islamabad (Capital of Pakistan). Almost all vegetables are grown in the pothwar region, but cucurbits are the main vegetables of the region. Cucurbits including cucumber (*Cucumis sativus* L) is grown in the field as well as in greenhouse throughout the year. Cucumber is thought to have originated in India, where it has been cultivated for the last 3000 years. [1,2]. It also has a long history in China, which is regarded as its secondary source. Cucumber tastes good and is free of fat, free of cholesterol and free of sodium. 100 grams of fruit contain 96.3 g of moisture, 0.4 g protein, 0.3 g minerals (140 mg of calcium, 30 mg of phosphorus and 0.6 mg of iron), 0.4 g of fibre, 5.7 g of carbohydrates, 0.04 mg of riboflavin, 0.4 mg of niacin and 4.0 mg of vitamin C. Cucumber is widely grown throughout the world, especially the slicing types. China is the largest producer of cucumbers and produces 51% of the world's total production [3]. In Pakistan, 3499 ha of cucumber is grown with an annual production of 40439 tons (FAO, 2013). The low production is attributed to biotic and abiotic factors and lack of resistant varieties. Plant viruses are known to cause devastating, and enormous losses and reduce crop quality and quantity [4]. One of these viruses is the Cucumber mosaic virus (CMV), which poses a major threat to the production of cucumbers in this region. The symmetry of CMV is isometric and infect a large number of plant species than any other plant virus [5,6]. In the temperate areas of the world, some monocotyledonous and many dicotyledonous plants are the primary host of CMV [7]. About 20 economically most important viruses have been detected from cucumber (*Cucumis sativus* L.) so far [8]. CMV infect 1287 plant species including cucurbits, solanaceous, cereals, vegetables, fruits, ornamentals [9]. Cucumber mosaic virus (CMV), a multi-component virus consisting of three genomic RNAs, each encapsidated in isometric particle diameter of 28 nm, is the species of genus *Cucumovirus* in the *Bromoviridae* family [10]. CMV has plus-sense single-stranded three segments of RNAs (RNA1, RNA2 and RNA3) having length of 3.4, 3.0, and 2.2 kb respectively along five open reading frames (ORFs) [11]. CMV's coat protein (CP) declared as a fundamental determinant transmitted by aphid vector [12]. Fny isolate of CMV is efficiently transmitted by *Myzus persicae Sulzer* and *Aphis gossypii Glover* [13]. Disease epidemics develop through primary inoculum. The main source of primary inoculum is weed host in which virus persist throughout the season [14]. In some crop, the virus is transmitted through planting materials, seeds and through weeds [15]. CMV is a notorious virus and presents worldwide especially in Pakistan. Prevalence, distribution and Coat protein (CP) base identification of CMV infecting cucumber in the pothwar region was the core interest of this study.

Materials and Methods

Survey and sample collection

The Pothwar region, with coordinate's latitude 32° 10 to 34° 9 N and longitude 71° 10 to 73° 55 E, is in the north-eastern part of Pakistan and it encompasses four districts namely, Chakwal, Attock Jhelum, Rawalpindi and a capital territory Islamabad. The survey was conducted during 2015- 16 in Pothwar region of Pakistan. The samples were collected from three to five randomly selected cucumber fields of

approximately 1 kannal. from each location. Viral symptoms, a variety of plant and crop stage have been recorded during the survey. Virus and virus-like symptoms like mosaic pattern, chlorosis, chlorotic streaks, interveinal chlorosis crinkling of leaf, puckering and malformed leaves exhibiting by leaf, as well as fruit were collected. The individual samples were preserved in labelled polyethene sample bags and these bags were put on ice packs in coolers while in transit. All the collected samples were brought to the Plant Virology laboratory in Plant Pathology department, PMAS Arid Agriculture University, Rawalpindi for coat protein base identification of virus by using serological and RT-PCR assay.

Serological and biological confirmation.

Preliminary detection of CMV was performed by using commercially available DAS-ELISA kit (Agdia Inc., Elkhart, IN) following manufacturer protocol for CMV [16]. The reaction was observed as strong (+++), moderate (++), mild (+) and weak (-) and intensity of yellow colour was measured as optical density (OD405nm) through ELISA reader (EPSON LX-300). CMV samples were considered as positive on the base of mean absorbance values at 405 nm that was double or more than the mean of the healthy control samples. Disease incidence was determined on the base of ELISA positive samples. The relative incidence was calculated using the following equation. (Steel and Torrie, 1980).

% D.I. of CMV=(No. of ELISA+ive samples)/(total No. of tested Samples) × 100

For biological confirmation test plant i.e., *Capsicum annuum*, *Cucumis sativus* cv, *Chenopodium amaranticolor*, C. *quinoa*, *Nicotiana tabacum*, and *Datura stramonium* were grown in the isolated condition in glasshouse by maintaining the temperature at 28 ± 2°C. When the plant was grown up to 2-3 leaf stage they were inoculated. For the preparation of inoculum 1 g of infected leaves were ground in an autoclaved mortar with pestle by adding 1 ml of 0.02 M phosphate buffer (pH 7.2). Crushed sap was passed through double layer muslin cloth. For causing minute injury 600-mesh carborundum powder was lightly dusted on indicator plant and sap was gently rubbed with forefinger on dusted leaves. After 10 min superfluous inoculum was rinsed with tap water and maintained these plants in a glass house for symptoms expression. After 2-3 weeks of inoculation, symptoms appeared on test plants.

RNA extraction and cDNA synthesis

TRIzol® (Life Technologies, Carlsbad, USA) reagent was used by following the manufacturer's protocol for total RNA extraction from ELISA positive samples of leaves as well as fruit. Quantification of RNA was done by Nanodrop (Thermo Scientific Co. Germany). 500 ng per µl of RNA working dilution in DNase/RNase free water was prepared. After RNA extraction Complementary DNA (cDNA) was manufactured. For synthesizing cDNA, RNA sample 5 µL, RNase free water 6 µL and 1 µL of reverse primer CMVR-45 5'-CCC CGG ATC CTG GTC TCC TT -3' having a molarity of 20 pM were used. (**Chen, 2003**), and incubation was done at 65°C for a period of 5 min leading to quick ice chilling.

After ice chilling 1 µL dNTPs (10 Mm), 5x Reaction buffer having quantity of 4 µL followed by 1 µL of Ribolock RNase inhibitor and 1 µL of Revert Aid Reverse Transcriptase was added and incubated at 42°C for a period of 60 min and 70°C for 10 min.

PCR amplification of CP gene

Amplification of the CP gene was done in PCR reaction mixture of 50 µL. Reaction mixture comprised of forward and reverse primer (1 µL of 20 pM each primer); forward CMVF-45 5'-CCC CGG ATC CAC ATC AYA GTT TTR AGR TTC AAT TC-3' and reverse primer; CMVR-45 (sequence mention earlier), 1 µL of dNTPs (10 Mm), 5 µL PCR buffer, 3 µL of $MgCl_2$ (25 mM), 0.4 µL Taq polymerase (500 u), nuclease-free water 35 µL and 4 µL of complementary DNA template. For performing PCR assay conditions was as follow: initial denaturation was performed at 94°C for 4 min (1 cycle), followed by 35 cycles comprises of Denaturation at 94°C for period of 1 min followed by annealing at 52°C for 1min and extension of 1 min at 72°C and final extension at 72°C for 5 min. Assessment of PCR product was carried out on 1.0% (w/v) agarose gel with 100bp DNA ladder in gel documentation system. Staining of agarose gel was done with 100 µg/mL of ethidium bromide.

Results and Discussion

To evaluate the incidence and distribution of CMV in Pothwar region surveyed was conducted during 2015-16. In survey 40 fields were visited throughout the growing season and 150 plant samples were collected. All the collected samples were tested through serological assay (Double antibody sandwich- Enzyme-linked immunosorbent assay) (DAS- ELISA) for the presence of *Cucumber mosaic virus* (CMV) [16]. Serological results depict that not even a single district of Pothwar region was found to free of CMV infection (Table 1). Leaf, as well as fruit samples such as mosaic, chlorosis, interveinal chlorosis, chlorotic streaks, gave ELISA positive results (Figure 1).

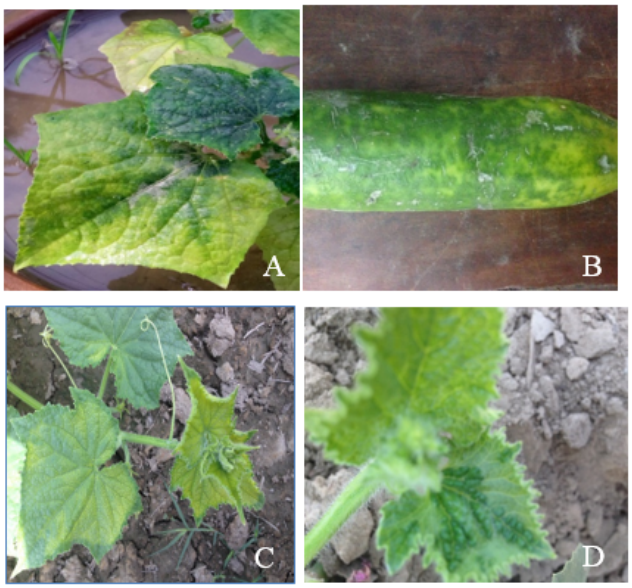

Figure 1: Leaf and fruit samples showing A) Mottling, B) Mosaic, C) Chlorosis and D) Puckering symptoms caused by CMV.

Coat protein (CP) of CMV is an important factor for inducing mosaic symptoms in an infected plant. The amplification of the CP gene by using a gene-specific primer from the sample showing mosaic symptoms agreed with the results reported by Green et al. [17]. CMV

has a broad host range So Green et al. [17,18], reported these symptoms from *Nicotiana tabacum* that coat protein gene of CMV is responsible for causing such symptoms along with other protein. In the present study 61 samples found to be ELISA positive (Table 1).

Location	No of samples +ve / No of sample tested	ELISA Reading for CMV	Percentage infection
Islamabad	12/30	1.152	40%
Rawalpindi	15/30	1.198	50%
Chakwal	14/30	1.173	46%
Jhelum	11/30	0.998	36%
Attock	13/30	1.165	43%
Total	65/150	-	-
Mean	13/30	1.1372	43

Table 1: Percent disease incidence of CMV and ELISA reading during 2015-16.

Disease incidence was higher in Rawalpindi (50%) followed by 46% in Chakwal, 43% in Attock, 40% in Islamabad and 36% in Jhelum. Similar disease incidence is also reported by Akthar et al. 2012. CMV has been reported in different ecological zones throughout the country. Its persistence is attributed to its inoculum which remains in the field for a long period of time. Shifting of inoculum from one location to another is due to the transportation of contaminated field equipment and due to human activities. Wind and viruliferous aphids are responsible for spreading of the virus to the long area. Similar observations have also been monitored by Grube et al. [7,19,20].

The reaction of tested plant

A sample of infected cucumber crop collected from different localities of Pothwar region during 2015-2016 showing virus-like symptoms when inoculated on tested plant i.e *Chenopodium amaranticolor*, C. *quinoa*, *Nicotiana tabacum*, *Cucumis sativus* cv, *Capsicum annuum* cv, *Datura stramonium*, shown symptoms describe in Table 2.

Test plant	Symptoms
Chenopodium amaranticolor	CL
Chenopodium. quinoa	NL
Nicotiana tabacum	#
Cucumis sativus	M, LD, ST
Capsicum annum	#
Datura stramonium	S
Symptoms key: CL=Chlorotic lesion, NL=Necrotic lesion, #=No disease symptoms appear, M=Mosaic, ST=Stunting, S= Spots	

Table 2: Symptoms shown by tested plant after mechanical inoculation with CMV.

Our results of indicator plants also agreed with results investigated by the previous researchers [21-24]. Control of virus can be achieved by eradication of the weeds by manually or by using herbicides. However, viruses may persist for a long time in these weeds. Seeds of annual weed, i.e., *Stellaria media* (Chickweed) are also a major cause of spreading of some viruses such as CMV [25]. No systematic work has been carried out in Pakistan to determine the loss of yield caused by viral diseases in the cucumber crop. CMV is one of the world's major virus - causing diseases, including Pakistan [26-28]. The expression of symptoms is usually not an accurate indication of the identity of the virus and should be treated with caution. New specific, efficient, sensitive and more reliable molecular tools (PCR) hae been introduced and CP gene-specific sense and antisense primer successfully amplified the virus. In this study few symptoms free and ELISA negative plants also produced an expected band through PCR amplification, but no band was observed in the negative control. However, the rapid development of the virus and the continued development of recombinant strains require an update of these tests at the nucleotide level [29]. The primer pair CMVF-45/CMVR-45 [26] amplified the expected DNA fragments of ~500 bp (Figure 2).

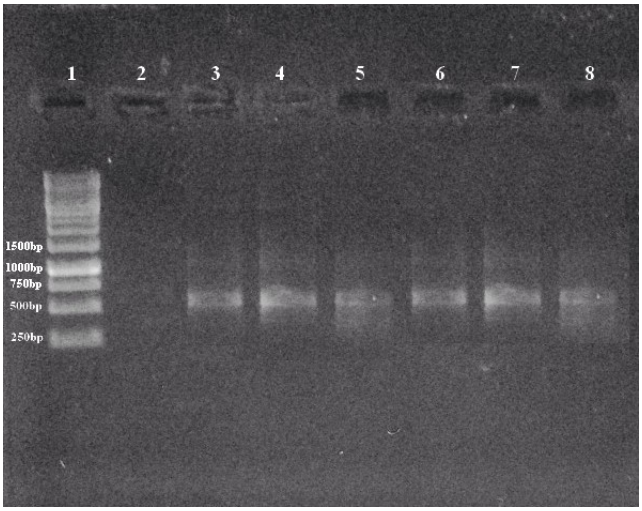

Figure 2: The gel showing the amplified band of ~500 bp corresponding to the CP gene.

The *cucumber mosaic virus* is one of the most important viruses known to have a wide range of hosts, so it is not easy to control.

Conventional measures such as cross-protection, eradication of infected plants, crop rotation, use of virus-free plants and use of chemicals against vectors have been used to control plant viral diseases for a long time [30]. But the chemical which are used to control the plant viral vector has adverse environmental effects and these chemicals also target the useful microorganism. Application of these chemical for long time, targeted insect pest develop resistance against these chemicals [31]. Anyhow, the use of resistant varieties is regarded as an economical and sustainable method of controlling viral diseases, and viral disease management has always preferred insect vector control and the use of resistant varieties. With the advancement of new technologies like use of nanoparticles and RNAi we can manage viruses. RNAi, a conserved eukaryotic mechanism, is involved in the growth, development, and host defense against viruses and transposons, that can also be hijacked to target insects, fungi, viruses, and weeds [32-37]. The emerging challenges faced by human being and food security shows clearly that there is an increasing demand and need in the agriculture sector to produce more output with little input. So, we are on the brink of adopting modern farming techniques and new innovative technologies to better and more precisely control these threats, as conventional agricultural practices will not be able to adequately control these threats without putting human health at critical risk. Among the latest technical approaches in the field of agriculture and to control plant disease, nanotechnology holds a prominent position in refurbishment of agriculture and controlling plant microbes which depict positively on the level of a healthy food production and combating the demands in a cost-effective and efficient manner.

Conclusion

It is concluded from the present findings that CMV was found in all district as well as in capital territory of Pakistan. This virus is a major threat to vegetable production in this region. There is need to evaluate this notorious virus at the molecular level through advanced molecular technologies and there is need to develop resistance cultivar against this high devastating virus for better production. During study virus is also identified from weeds plant which indicate that these weeds plant provide shelter to CMV so there is need to eradicate these weeds from fields. It is also observed that insect vector transmit virus efficiently through long distance so there is need to focus on controlling the insect vector that transmits virus at long distance.

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