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Original Article

Molecular diversity and phylogenetic reconstruction of *Pepper mild mottle virus* isolates from Pakistan

Adnan Ahmad[1*], Muhammad Naveed Aslam[1], Fasiha Qurashi[2], Waqas Ashraf[1], Muhammad Raheel[1], Qaiser Shakeel[1], Ambreen Maqsood[1], Kamran Saleem[3]

[1]Department of Plant Pathology, Faculty of Agriculture and Environment, The Islamia University of Bahawalpur, 63100, Bahawalpur, Pakistan

[2]Department of Forestry, Range and Wildlife Management, Faculty of Agriculture and Environment, The Islamia University of Bahawalpur, 63100, Bahawalpur, Pakistan

[3]Plant Protection Division, NIAB, Faisalabad, Pakistan

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**Corresponding author email:*
adnan_84@outlook.com

Abstract

Pepper mild mottle virus is a lethal *Tobamovirus* infecting capsicum around the globe. Molecular diversity of capsid protein gene (CP) of Pakistani *Pepper mild mottle virus* (PMMoV) isolates was investigated. From symptomatic pepper leaves collected from farmer's fields, the CP gene of PMMoV RNA was amplified by specific primers designed in this study. The nucleotides sequences of Pakistani PMMoV isolates were 98.2% to 99.3% similar to each other and 97.2% to 99.3% with other isolates. Highest identities were observed with Indian (NC-3) and Chines (C27084) isolates. In phylogenetic reconstruction, Pakistani isolates grouped with Turkish and South Korean isolates. Few single nucleotide polymorphism were detected in Pakistani isolates and no insertions or deletions were observed. There was 0.0020 to 0.0063 evolutionary distance among Pakistani isolates and 0.0021 to 0.018 between Pakistani and world isolates (highest with Indian and German, and lowest with Spanish, Chines and Brazilian isolates). A frequent gene flow (Fst = 0.07103 i.e. <0.33) was observed between Pakistani and world isolates. In investigation of genetic differentiation, the figures of permutation-based statistical tests viz; Z (296.07432), Snn (0.083571) and Ks* (1.36036) were significant. In statistical analysis the values of Fu & Li's D* and F* and Tajima's D, were negative, exhibiting the low polymorphism frequency in studied populations.

Keywords: Tobamoviruses, *Pepper mild mottle virus*, Molecular diversity, CP gene

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Introduction

Capsicum is one of the most extensively consumed spices and vegetables around the world. Pakistan is the 4th largest producer by sharing 4% to the global capsicum production (Arain, 2019). About 65 viruses infect capsicum worldwide and the *Pepper mild mottle virus* (PMMoV) (Genus: *Tobamovirus*, Family:

Togaviridae) is one of the four lethal capsicum infecting tobamoviruses (TMGMV, ToMV, TMV, PMMoV) (Ikegashira et al., 2010). The virus is seed-borne and globally distributed (Velasco et al., 2002; Fauquet and Fargette, 2005). It survives by remaining inactive for years inside infected plant debris and soils that provide primary source of inoculum to cause infection in succeeding crops (Adams et al., 2016). Instead of insects, PMMoV externally spreads by soil, seeds and mechanical ways like pruning and transplant (Ikegashira et al., 2010). There are no reports of PMMoV infection in tobacco, tomato and eggplant (Velasco et al., 2002). About 300 nm long and 18 nm helical cylindrical non-enveloped virion of PMMoV contains a 64kb monopartite +ssRNA that codes for nucleoprotein, 130 K protein, a movement protein and 180 K replication protein (Lamb et al., 2001). PMMoV induces mild foliar chlorosis, puckering, yellowing, stunting and undersized malformed fruits with mottling and streaks on mature ovaries along with sunken or raised necrotic spots (Genda et al., 2005).

Accurate identification of plant viruses is crucial for the development of sustainable management strategies like resistant varieties. Moreover, the molecular characterization is currently the most extensively used tool to classify viral strains, species or variants. Molecular data of most of the Pakistani plant viral isolates is unexplored and insufficient to develop sustainable management strategies. So, the present study was aimed to detect, identify and characterize the Pakistani PMMoV-isolates based on identity percentages, phylogeny and molecular diversity of CP gene sequences.

Material and Methods

Sample collection

During field surveys in Punjab province of Pakistan in 2018, the symptomatic capsicum leaves were collected from farmer fields and transported to laboratory in zipper bags stored in ice.

Bioassay

The PMMoV isolates were biologically purified by local lesion assay performed on *Chenopodium quinoa* plants. Also, the viral inoculum was propagated on *Capsicum annum*, *Nicotiana tabaccum* and *Datura metal*. Three consecutive mechanical inoculations were done by applying the capsicum leaf sap (Homogenized in 0.1 M phosphate buffer) on leaves of above-mentioned host plants at 5-6 leaf stage. Leaves were pre-dusted with 600 mesh carborandom (Mnari-Hattab and Ezzaier, 2006). Inoculated plants were kept in glasshouse at 25^{o}C and ~70% relative humidity, and were observed (for local lesions and/or symptoms) after one to two weeks of inoculation.

RNA isolation, cDNA synthesis and PCR amplification

Total RNA was isolated by TRIzol® Reagent method. The cDNA of viral RNA was synthesized by virus specific reverse primer using Thermo Scientific RevertAid First Strand Synthesis kit as per manufacturer's instructions and capsid protein gene was amplified by using primers; PMMoVCP-F 5'-GAGGAAGCTGGTTGACAAGG-3' and PMMoVCP-R 5'-CGTTCGCAAATACACGTCAC-3' in PCR using Thermoscientific GreenTaq PCR Master Mix at following temperature conditions; 94°C for 2 minutes and 35 cycles of 94°C, 55°C for 30 seconds and 72 for 45 seconds followed by a final extension at 72°C for 10 minutes. The amplified nucleic acid fragments were separated in 1% (w/v) agarose gel containing 100 µg/mL EtBr and visualized in UV light. Purification of PCR products was done by QIAquick® PCR purification kit (Qiagen) and dually sequenced by Macrogen, Korea.

Sequence analysis

Protein translation of obtained nucleotide sequences was done by EMBOSS Transeq program (Rice et al., 2000) and subjected to BLAST analysis. The resembling PMMoV CP gene sequences from NCBI database were downloaded and aligned by using CLUSTAL W program (Larkin et al., 2007). Calculation of evolutionary distance and phylogenetic reconstruction were performed by MEGA 10 software using maximum likelihood method and 1000 bootstrap replicates with default parameters (Tamura et al., 2013).

Selection pressure and recombination analysis

Number of insertion and deletion events, single nucleotide polymorphism, nucleotide and haplotype diversities in all sites and molecular diversity pattern at segregating sites by statistical analysis like Fu, & Li's F*, Fu, & Li's D* and Tajima's D, were recorded by 5.0 version of DnaSP software (Librado and Rozas, 2009). Recombination detection program version 4.0 was used to detect possible recombination events in nucleotide sequences.

Results

Biological purification

Mechanically inoculated *Chenopodium quinoa* plants produced localized necrotic lesions. The indicator host plants viz; *Capsicum annum* cv. Sanum and cv. Loungi, *Nicotiana tabaccum* and *Datura metal* clearly exhibited foliar chlorosis, puckering and yellowing symptoms (Fig. 1) that are typically associated with PMMoV infection. The PMMoV infection in mechanically inoculated plants was verified by RT-PCR.

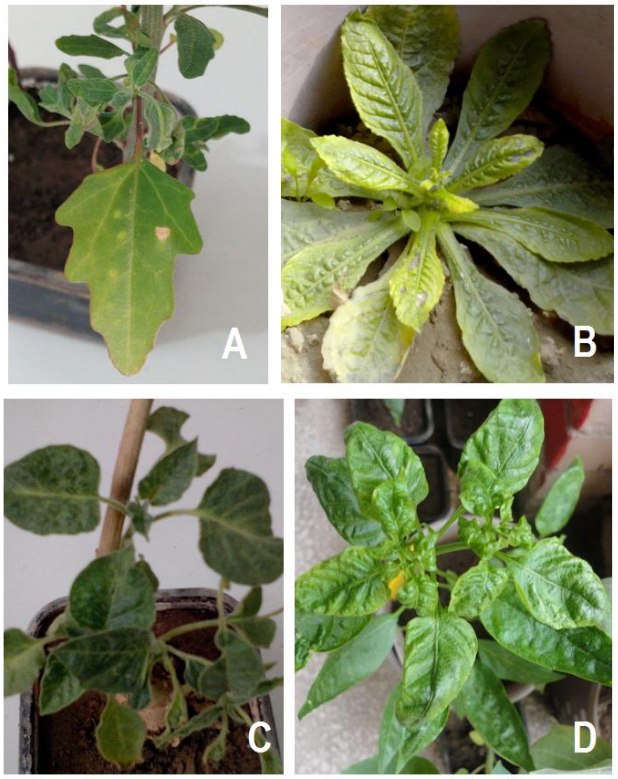

Figure-1. Mechanically inoculated *Chenopodium quinoa, Nicotiana tabaccum, Datura metal* and *Capsicum annum* plants showing typical symptoms.

PCR amplification and sequence analysis

The DNA fragments of ~700 base pairs were amplified by PCR. After trimming the obtained sequences, 574 nucleotides containing 471 nucleotides of complete CP gene (encoding for 157 amino acids and located between 5685th to 6158th nucleotide of PMMoV genome) and 103 nucleotides of 3'UTR were acquired from each PCR product. These sequences were submitted in GenBank (Table 1). Each of these sequences was comprised of 18 to 19% Guanine, 21 to 22% Adenine, 33 to 34% Uracil and 27 to 28% Cytosine, (Table 1). Sequences were aligned to examine the variations (Fig. 2). BLASTn analysis recorded 98.2 to 99.3% nucleotide and 95.7 to 97.4% amino acid similarities among Pakistani isolates. While, they shared 97.2 to 99.3% nucleotide and 92.7 to 97.8% amino acid identities with world isolates (Table 2). Highest identities were observed with Indian (isolate NC-3) and Chines (isolate C27084) isolates. In a maximum likelihood phylogenetic reconstruction, Pakistani isolates grouped with Turkish and South Korean isolates in a close association with Brazilian isolate (Fig. 3).

Table-1. Characteristics of nucleotide sequences of Pakistani PMMoV isolates submitted in GenBank.

Isolate	Accession Number	Viral cDNA (nt)	Coat Protein Gene						3'NTR
			(nt)	(aa)	A %	C %	G %	U %	(nt)
PMMoV PK1	MN966570	574	471	157	34	20	24	22	103
PMMoV PK2	MN966571	574	471	157	33	20	24	23	103
PMMoV PK3	MN966572	574	471	157	34	20	23	23	103
PMMoV PK4	MN966573	574	471	157	33	20	24	23	103
PMMoV PK5	MN966574	574	471	157	33	20	24	23	103

Table-2. Sequence characteristics, selection pressure and gene flow analysis of Pakistani PMMoV isolates.

Analysis	Values
Nucleotide identities (with each other)	98.2-99.3%
Nucleotide identities (with other isolates)	97.2-99.3%
Amino acid identities (with each other)	95.7-97.4%
Amino acid identities (with other isolates)	92.7-97.8%
Evolutionary distance among Pakistani isolates	0.0020-0.0063
Evolutionary distance with other isolates	0.0021-0.018
INDELs	No
Fst	0.07103
Fu, & Li's D*	-4.34614
Fu, & Li's F*	-4.34706
Tajima's D	-2.34630
Ks*	1.36036
Z	296.07432
Snn	0.083571
Recombination	No

Figure-2. Amino acid sequence alignment of Pakistani *Pepper mild mottle virus* isolates.

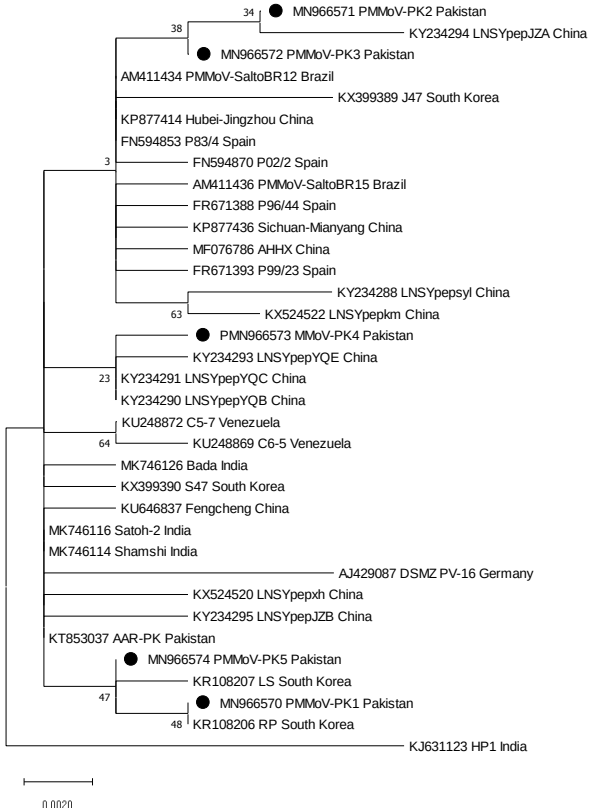

Figure-3. A 1000 bootstrap consensus tree based on nucleotide sequences of CP gene of five PMMoV isolates from Pakistan (dotted) and 30 database sequences.

Selection pressure and recombination analysis

The nature of polymorphism between PMMoV isolates was detected by examining the number of insertions and deletions (INDEL) and single nucleotide polymorphism (SNP) in their nucleotide sequences. A total of 2, 3, 2, 2 and 2 SNPs were recorded in PMMoV-1 to PMMoV-4 isolates respectively, and no INDEL were detected. Evolutionary distance among Pakistani isolates was 0.0020-0.0063 and 0.0021-0.018 with world isolates (Table 2). The evolutionary distances were highest with KJ631123 and AJ429087 isolates as 0.014-0.018 and 0.010-0.014 and lowest as 0.009-0.013 with FN594853, KP877414 and AM411434 isolates.

Gene flow and genetic differentiation analysis

Gene flow analysis revealed 0.07103 value of coefficient of gene differentiation (Fst) which is less than 0.33 standard value that suggests the gene flow to be frequent between Pakistani and world isolates. Investigation of genetic differentiation also revealed the figures of permutation-based statistical tests viz; Z (296.07432), Snn (0.083571) and Ks* (1.36036) as significant (Balasubramanian and Selvarajan, 2014). In statistical analysis the values of Fu & Li's D* and F* and Tajima's D, were found negative as, -4.34614, -4.34706 and -2.34630 respectively (Table 2). This shows low polymorphism frequency in PMMoV population (Balasubramanian and Selvarajan, 2014).

Discussion

Molecular characterization based on CP gene of several viruses including PMMoV has been studied worldwide (Antignus et al., 2008; Ahmad and Ashfaq, 2018; Çağlar et al., 2013), as this gene holds key importance in viral classification, life cycle of plant viruses, synthesis of capsid protein, and host pathogen interaction (Moury and Simon, 2011). According to ICTV rules, there exists more than 80% amino acid and nucleotide sequences homology in CP genes of same species (Adams et al., 2016). In current study, all of the Pakistani PMMoV CP gene sequences shared more than 80% and 90% amino acid and nucleotide sequences homologies with global *Pepper mild mottle virus* (PMMoV) isolates, which verifies their taxonomic status. Moreover, in collected samples the presence of *Pepper mild mottle virus* (PMMoV) symptoms as reported by numerous researchers around the globe also supports the accurate identification of this virus (Sevik, 2011). The *Pepper mild mottle virus* (PMMoV) was first time reported from Pakistan in 2015 infecting capsicum. However, its incidence

and distribution across the country is unknown. The nucleotide and amino acid sequence analysis of CP gene of PMMoV shows that most of the Pakistani isolates shared higher identities and clustered with Indian and Chines isolates. Beside, mechanical means like routine handling especially at transplanting, pruning and harvesting (Ikegashira et al., 2010) the tobamoviruses are transmitted over long distances by infected plantlets and seeds (Svoboda et al., 2006). Pakistan buys most of its vegetable and major crop seeds from other countries like, India and China. The quarantine measures adopted by Pakistan are not enough to check the movement of plant pathogens (Tsompana et al., 2005).

In this study, a frequent gene flow among Pakistani and world PMMoV populations was also observed. The negative values of neutrality tests like Tajima's D, Fu, Li's D*, and Fu, Li's F* also indicate the PMMoV population under negative or purifying selection. Evolutionary distance describes the divergence of homologous sequences from their common ancestors (Rosenberg, 2005). In present research, the higher sequence identities, close phylogenetic relationship and lower evolutionary distance of Pakistani isolates with India and China express their geographical association.

Conclusion

The above-mentioned findings and discussion suggest that the CP gene nucleotide sequences of Pakistani PMMoV isolates hold lower molecular diversity as revealed by purifying selection and frequent gene flow, and they share higher sequence identities and phylogenetic association with Chines and Indian isolates. Hence, a strict quarantine is suggested on the movement of seeds and other planting material across the border. Moreover, comprehensive surveys for detection and molecular characterization based on complete genomic sequence of this virus from capsicum as well as other crops is also recommended to develop sustainable and comprehensive management strategies like development of resistant or tolerant varieties by biotechnological approaches.

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Contribution of Authors

Ahmad A: Conceived idea, conducted experiments and article write up
Aslam MN: Field surveys and laboratory analysis support
Qurashi F: Conducted experiments and article write up
Ashraf W: Field surveys and article write up
Raheel M: Designed research methodology & data collection
Shakeel Q: Data analysis and article write up
Maqsood A: Lab experiments and writeup.
Saleem K: Final editing and approval of article