Pak. j. life soc. Sci. (2017), 15(2): 84-89 E-ISSN: 2221-7630;P-ISSN: 1727-4915

Pakistan Journal of Life and Social Sciences
www.pjlss.edu.pk

RESEARCH ARTICLE

Alternanthera Yellow Vein Virus (AYVV) Infecting *Eclipta prostrata* Plant is Associated with Two Novel Alphasatellites in Pakistan

Ghulam Murtaza[1], Muhammad Mubin[1*], Muhammad Shah Nawaz-Ul-Rehman[1] and Luqman Amrao[2]

[1]Virology Lab, Center of Agricultural Biochemistry and Biotechnology, University of Agriculture, Faisalabad, Pakistan

[2]Department of Plant Pathology, University of Agriculture, Faisalabad, Pakistan

ARTICLE INFO

Received: May 05, 2017
Accepted: Jul 09, 2017

Keywords
Alphasatellites
Begomoviruses
Eclipta prostrata
Recombination
Recombination Detection Program (RDP)

***Corresponding Author:**
mmubin@uaf.edu.pk

ABSTRACT

Whitefly-transmitted begomoviruses (Family *Geminiviridae*; genus *Begomovirus*) are known to occur in the Indian subcontinent for a long time but recently emerged as major pathogens on food and fiber crops. During a field survey in 2012-13, *Eclipta* (*E.*) *prostrata* leaf samples showing vein-yellowing symptoms were collected from two different districts of the Punjab, Pakistan. Amplification of the full-length viral molecules and associated satellite molecules was performed through rolling circle amplification (RCA) and polymerase chain reaction (PCR). Cloning, sequencing and sequence analysis of full-length virus and associated satellites showed *E. prostrata* as a source of multiple and diverse alphasatellite molecules associated with Alternanthera yellow vein virus (AlYVV). AlYVV was a recombination-free virus, showing 95% sequence homology with AlYVV from China. *E. prostrata* carried two different alphasatellite molecules i.e. Tomato yellow leaf curl China alphasatellite (TYLCCNA^{Ecl}) and a new specie, Eclipta yellow vein alphasatellite (ECYVA). TYLCCNA^{Ecl} showed 88% sequence homology to Tomato yellow leaf curl China alphasatellite (TYLCCNA) while ECYVA showed 72% sequence homology with Holly-hock yellow vein alphasatellite. There was no betasatellite molecule found to be associated with vein yellowing disease of *E. prsotrata*. The TYLCCNA^{Ecl} was a recombinant molecule with recombination of alpha-rep region from TYLCCNA, while A-rich region from Tobacco curly shoot alphasatellite. This is the first report of a recombinant alphasatellite. The other molecule ECYVA did not show any level of recombination with other alphasatellites. The data presented here would help in understanding the role of weeds in speedy evolution of begomoviruses associated satellites.

INTRODUCTION

Begomoviruses (family *Geminiviridae*) are the most widespread single-stranded DNA (ssDNA) viruses in Pakistan. Many economically important crops like Cotton, Potato, Tomato and Chilies are infected by begomoviruses (Juarez et al., 2013; Mazhar et al., 2000; Mubin et al., 2009a; Tahir and Haider, 2005). The recent global spread of whitefly has resulted in more epidemics and several new begomovirus disease complexes have been found to infect crops. Little information has been reported so far regarding infection of begomoviruses and associated satellites on weeds. Weeds serve as alternative host for crop-infecting begomoviruses, when main cropping season is not there. Different begomoviruses and associated satellites namely, alphasatellite and betasatellite (Briddon et al., 2003; Briddon et al., 2004) infect weeds, like *Croton bonplandianus*, *Sonchus arvensis* and *Alternanthera* (Hussain et al., 2011; Guo and Zhou, 2005; Mubin et al., 2009b; Mubin et al., 2010; Mubin et al., 2012). Betasatellites are pathogenicity determinant molecules of disease complex while the role of alphasatellites is still unclear.

Alphasatellites are begomoviruses associated satellite molecules, dependent on helper virus for movement and transmission to the other plant hosts (Briddon et al., 2004). Alphasatellites are 1300-1400 nucleotides long

single stranded circular molecules. These molecules are emerging as the most diverse satellites in crop plants and weeds both from New World and Old World (Briddon et al., 2004; Fiallo-Olive et al., 2012). The origin of alphasatellites is traced back to nanoviruses (another class of ss DNA viruses) (Briddon et al., 2004). The genome of alphasatellites is composed of a Rep gene, known as alpha-rep, A-rich region and origin of replication similar to nanoviruses (Briddon et al., 2004). The role of alphasatellites in the etiology of different diseases is unknown. These molecules are diverse in nature and do not play a role in the development of disease symptoms. The alphasatellites found from non-cultivated Cotton species were found to be suppressors of gene silencing (Nawaz-Ul-Rehman et al., 2010).

Alternanthera is a widely grown weed in India, Pakistan and China. Alternanthera was infected by a new species of begomovirus, namely Alternanthera yellow vein virus (AlYVV) in Hainan province of China during 2004 (Guo and Zhou, 2005). Since then AlYVV has been reported from *Eclipta* (*E*.) *prostrata* and several other weeds in different Asian countries (Mubin et al., 2010). AlYVV seems to be widespread begomovirus in Pakistan, India and China. However, none of the hosts have shown it to be associated with DNA-B component, suggesting it as a monopartite begomovirus. In Pakistan, AlYVV has been found to be associated with multiple satellites, like Ageratum yellow vein betasatellite and Potato leaf curl alphasatellite (Mubin et al., 2010). But these isolates were identified from *Sonchus arvensis* weed. While in China, no such satellites have been reported from Eclipta or Alternanthera plants. Similarly, in Vietnam also, there are no reports of alphasatellites or betasatellites in combination with AlYVV (Ha et al., 2008).

In this study, we have surveyed the *E. prostrata* plants for the possible presence of alphasatellites in combination with AlYVV. To our surprise, there were diverse alphasatellites associated with AlYVV in the region while no betasatellites were detected from these Eclipta plants. Our data suggests that AlYVV is a betasatellite independent virus associated with diverse alphasatellites.

MATERIALS AND METHODS

Virus sources and DNA extraction

The symptomatic plants of *Eclipta* (*E.) prostrata* showing vein yellowing were collected from different areas of Punjab from farmer's fields. The asymptomatic leaves were taken as negative control. Young leaves were collected, labeled and transported on ice to lab and stored at -80°C. Total DNA was extracted from leaf samples by CTAB method as described by Doyle and Doyle (1990).

Amplification of begomovirus and associated satellites

Total DNA extracted from infected leaves of *E. prostrata* was subjected to rolling circle amplification (RCA) (Blanco et al., 1989). The RCA product was restricted using different restriction enzymes i.e., S*alI, BglII, HindIII, KpnI* and *Sac*I. Fragments of size 2.8 kb, which could be begomovirus, were generated by restriction with *Sac*I enzymes. The restricted product was gel eluted and cloned into the pTZ57R/T vector (Fermentas) and completely sequenced. PCR was used to amplify begomovirus associated satellites as no 1.4 kb size fragment was obtained by restriction of RCA product. Briefly, 20ng/ul of total DNA was used in a 25ul reaction. The mixture included ingredients of PCR mix (Thermo scientific) along with dNTPs and Universal alphasatellite or betasatellite primers (Briddon et al., 2002; Bull et al., 2003). The full-length PCR products were resolved on the agarose gel and a band of 1.4 kb was confirmed. The PCR products were ligated into pTZ57R/T vector through direct T/A cloning procedure (Fermentas). Positive clones were sequenced through dideoxy method of sequencing.

Sequence analysis and recombination detection

Sequences were assembled and analyzed by the Lasergene DNA analysis package (v8; DNA Star Inc., Madison, WI, USA). Pairwise comparisons for sequence similarities were produced using the MegAlign program of the Lasergene package. Phylogenetic trees were generated, first by aligning the molecules using CLUSTAL-W, followed by the neighbor joint method of phylogenetic tree construction in MEGA7 program. The accession numbers for AlYVV, ChLCA and AlYVA are GenBank: KX906694-97, KX938426 and KX938425, respectively. The other alphasatellite sequences were downloaded from GenBank and virus abbreviations are used as described by ICTV. After the sequence confirmation, detailed recombination analysis was conducted for satellites using recombination detection program (RDP) (Martin and Rybicki, 2000). Prior to recombination analysis the sequences were aligned by Clustal-W in MEGA7 DNA analysis software followed by recombination detection through RDP3 program.

RESULTS

Symptomatology

Leaves from 15 *Eclipta* (*E.) prostrata* plants showing vein-yellowing symptoms, typical of begomovirus infection, were collected from two different districts of Southern parts of the Punjab province in 2012 and 2013 (Fig. 1A). In all these areas there were cultivated crops like Cotton, Rice, Sugarcane and vegetable crops present in the field. This weed is commonly found in the fields as well as around the water channels. At every place *E. prostrata* plants were found to be showing

Fig. 1: *Eclipta prostrata* plant; A) Symptomatic plant showing vein yellowing, B) asymptomatic plant grown in virus free conditions.

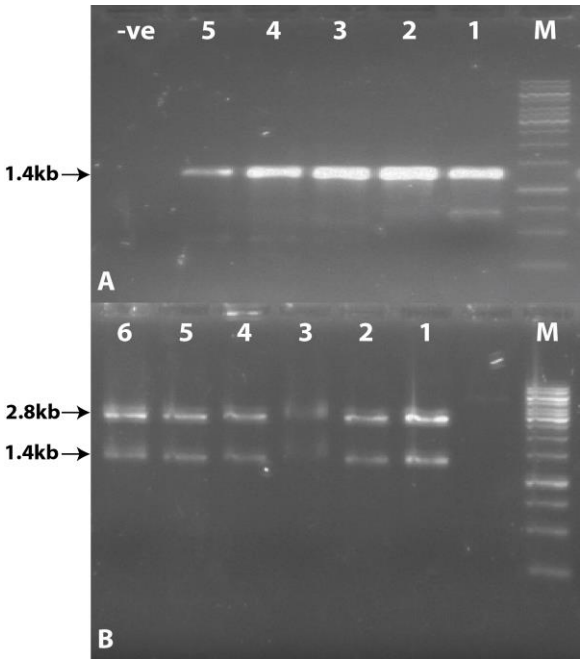

Fig. 2: PCR amplification from symptomatic leaves; A) Amplification of alphasatellite through PCR: 1 to 4 PCR amplifications from infected samples, lane 5 is positive control and lane 6 is negative control. M is the 1kb Marker, B) Lane 1-6, confirmation of 1.4Kb sized clones of alphasatellites by restriction with *EcoRI* and *PstI*.

similar symptoms of vein yellowing. Vein yellowing was so conspicuous that the infected plants showed clear distinction from the non-infected ones (Fig. 1B).

Cloning of begomovirus and alphasatellites from *E. prostrata*

Rolling circle amplification (Blanco et al. 1989) was used to amplify all circular ssDNA molecules from infected samples of *E. prostrata*. Restriction with *Sac*I enzyme yielded 2.8 kb fragment i.e., size of begomovirus and cloned in pTZ57R/T (gel pic not shown). The Universal alphasatellite primers (Bull et al., 2003) were used to amplify the molecules in a PCR reaction (Fig. 2A). The PCR products were amplified and cloned in pTZ57R/T vector. The restriction digestion with *EcoRI* and *Pst*I enzyme confirmed the 1.4kb sized bands in the gel (Fig. 2B). The 1.4kb sized bands confirmed the presence of alphasatellites in the clones. We were unable to amplify betasatellites by using the same dilution as a template in PCR reaction.

Sequence analysis of begomoviruses

A total of around 75 DNA A molecules of size 2.8 kb were cloned from fifteen plant samples collected from different regions of Punjab and partial sequencing showed that all molecules show maximum sequence similarity to Alternanthera Yellow Vein Virus (AlYVV). After partial sequencing, 4 molecules were completely sequenced. These completely sequenced molecules also showed that only AlYVV is present in all infected samples. Diagnostic primers for DNA-B were also used to find out the presence of any DNA-B molecule. There was no hint of the presence of DNA-B in any of these samples. These viruses isolated from *E. prostrata* showed the highest homology with the already reported AlYVV, although samples were taken from distant places of Punjab province.

Sequence analysis of alphasatellites

Total of 60 full-length alphasatellites were cloned and sequencing showed two different types of alphasatellites associated with AlYVV. These molecules were checked for identity percentage with other molecules in GenBank through BLAST. The BLAST results showed that the isolates obtained from Multan region (Accession no; KX938426) were closer to the alphasatellites from Chinese origin. Indeed, it showed 88% homology with Tomato yellow leaf curl China alphasatellites (TYLCCNA) (Table 1). In the phylogenetic tree of alphasatellites, the KX938426 made a cluster with Chili leaf curl alphasatellite (ChLCA) from Pakistan or India, Tobacco curly shoot alphasatellite (TbCSA) from China and TYLCCNA (Fig. 3). This close clustering shows that alphasatellite associated with eclipta yellow vein disease from Multan region is identical to TYLCCNA. Therefore, we proposed its name as an Eclipta strain of Tomato yellow leaf curl China alphasatellite ($TYLCCNA^{Ecl}$). This isolate had a typical genome organization, just like other alphasatellites from the Old World. It contained TAGTATTACC, as an origin of replication and 315 amino acids of its alpha-Rep.

The second alphasatellite molecule isolated from Layyah district showed only 47-72% sequence identity with all the alphasatellites in BLAST analysis. The detailed identity percentage of the isolate (KX938425) showed its maximum homology with Hollyhock yellow vein alphasatellite (HoYVA-72%) or Croton yellow vein mosaic alphasatellite (CYVMA-62%). Due to its less similarity with other alphasatellites, we proposed its name as Eclipta yellow vein alphasatellite (EcYVA). As compared to $TYLCCNA^{Ecl}$ it contained an Alpha-rep of 289 amino acids.

Table 1: Pairwise comparison of alphasatellites from the present study with selected alphasatellite molecules from data bank.

Virus name	TYLCCNA	AConSLA	ChLCuA	CLCuBuA	CLCuMuA	EcYVA	CYVMA	HHYVA	GDarSLA	TbCSA
TYLCCNA*-[KX938426]	88	72-73	83-88	72	88	50	52	51	53	88
AConSLA-[LN880827]		97	68-71	76	72	50	52	52	57	84
ChLCuA-[KF584013]			94	69	94	49	51	51	53	80
CLCuBuA-[HF567947]				96	72	49	54	51	56	72
CLCuMuA-[KJ028212]					98	49	54	51	56	72
EcYVA* [KX938425]						-	66	72	67	50
CYVMA-[LN879487]							90	71	92	53
HHYVA-[FR772086]								-	72	52
GDarSLA-[EU384652]									97	54
TbCSA-[KT390436]										84

*isolates from the present study

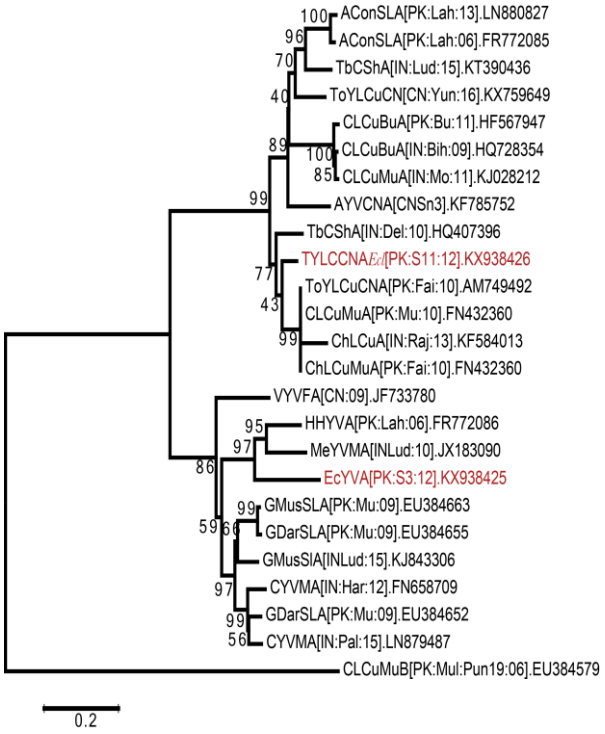

Fig. 3: Phylogenetic dendrogram based upon selected complete sequences of alphasatellites molecules. Alphasatellite sequences used for comparison are downloaded from databank. The database accession number in each case is given. The sequences associated with Alternanthera yellow vein disease are indicated by a red color in each case.

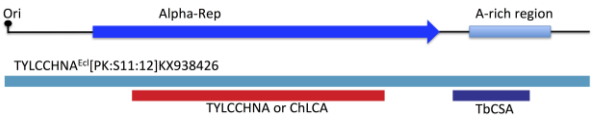

Fig. 4: Recombinant Alphasatellite TYLCCNA[Ecl]. Red colour segment shows the part of ChLCA or TYLCCNA and blue color shows a part from TbCSA.

Recombination analysis of alphasatellites

The recombination of alphasatellites isolated in this study and from GenBank was calculated through recombination detection program RDP (Martin and Rybicki, 2000). The isolate TYLCCNA[Ecl] (KX938426) showed recombination of alpha-rep region with TYLCCNA or ChLCA, while A rich region showed maximum identity with TbCSA (Fig. 4). The recombination was further confirmed by BLAST analysis. Indeed, it is the first report of a recombinant alphasatellite. While the EcYVA did not show any level of recombination with other alphasatellite.

DISCUSSION

The viruses belonging to genus *Begomovirus* are increasingly spreading to the weeds and cultivated crops (Mansoor et al., 2006). These begomoviruses are associated with satellites in India, Pakistan, China and several other Asian countries (Mansoor et al., 2003; Mansoor et al., 2006; Mubin et al., 2009b; Mubin et al., 2012). Previously, these satellites were only known from Old World, but recently due to efficient cloning techniques, they have also been reported from New World (Fiallo-Olive et al., 2012). Despite the fact that several begomoviruses and satellites are known from India and Pakistan, but still the complete diversity of molecules is still least understood (Vinoth et al., 2017). According to the pairwise sequence comparison analysis, 84% sequence similarity is the criteria to identify the alphasatellites species (Vinoth et al., 2017). Although, alphasatellites have not been classified yet, according to literature survey, 83 or 84% have been set as a criterion to identify the new species.

The yellow vein symptoms of *E. prostrata* have been attributed to geminiviruses infection previously (Zaidi et al., 2017; Ha et al., 2008; Haider et al., 2005). However, satellites have not been reported in combination with begomoviruses in Eclipta plant from Pakistan (Zaidi et al., 2017). We have identified two novel alphasatellites from two different locations in

Pakistan. There was no change in helper viral sequence in both the locations. The DNA-A components were typical of Alternanthera yellow vein virus (AlYVV, accession no. KX906694-97). The nucleic acid sequencing and then the BLAST information revealed that the full-length viral molecules (~ 2.8kb) from all different localities were found to be highly conserved and level of sequence homology was 92 to 98%. To our surprise, we were unable to find a single betasatellite from these samples. Therefore, we applied universal primers based PCR. We used both the genomic DNA dilutions and RCA dilutions to amplify any detectable level of betasatellite. But surprisingly, there was no amplification of betasatellite. This showed that betasatellites might not be associated with Eclipta yellow vein disease in *E. prostrata* plants. Similar results were shown by (Ha et al., 2008; Zaidi et al., 2017*)*, as they isolated the AlYVV from *E. prostrata* and Zinnia elegance.

The phylogenetic analysis of closely related alphasatellites showed two major clusters. One cluster showed higher nucleotide identity with alphasatellites associated with Tomato yellow leaf curl China Alphasatellites. Therefore, we proposed its name (KX938426) as Tomato yellow leaf curl China alphasatellite-Eclipta strain (TYLCCNA^{Ecl}). Recombination analysis of this isolate revealed a unique pattern (Fig. 4). The alpha-rep showed close similarity with TYLCCNA or ChLCA, while the A-rich region showed maximum homology with TbCSA. According to our literature survey, there is no such example of alphasatellites recombination.

Due to less than 84% identity with other alphasatellites in the GenBank the isolate KX938425 was considered as a new specie. Hence, its name was proposed as *Eclipta yellow vein alphasatellite* (EcYVA). In the phylogenetic tree, EcYVA made a separate cluster with *Gossypium darwinii* symptomless alphasatellite (Fig. 3), which were originally isolated from non-cultivated cotton species (Nawaz-ul-Rehman et al., 2012).

There are many interesting findings presented here. Usually in old world, begomoviruses are associated with betasatellites but in this study no associated betasatellite was found, though association with multiple alphasatellites was found. AlYVV was found to be recombination free across the infected samples collected from Punjab, which again is an interesting observation. In summary, the present study reveals that Alternanthera yellow vein disease in Pakistan is associated with novel and multiple alphasatellites.

Authors' contribution

GM performed all the experiments. MM, MSN and LA were involved in design of the experiments and data analysis. MM wrote the paper while MM, MSN critically reviewed the manuscript.

Acknowledgments

Higher Education Commission of Pakistan supported Mr. Ghulam Murtaza under 5000 Indigenous Ph.D. fellowship program. This work was funded by the IFS Research Grant No. C/5260-1 to Dr. Muhammad Mubin and IFS Research grant No. C/5434-1 to Dr. Shah Nawaz ul Rehman. This work is part of PhD research thesis of Mr. Ghulam Murtaza. We acknowledge all members of virology lab for help and support to perform experiments for this work.

REFERENCES

Blanco L, A Bernad, JM Lazaro, G Martin, C Garmendia and M Salas, 1989. Highly efficient DNA synthesis by the phage phi 29 DNA polymerase. Symmetrical mode of DNA replication. Journal of Biological Chemistry, 264: 8935-8940.

Briddon RW, SE Bull, S Mansoor, I Amin and PG Markham, 2002. Universal primers for the PCR-mediated amplification of DNA beta: a molecule associated with some monopartite begomoviruses. Molecular Biotechnology, 20: 315-318.

Briddon RW, SE Bull, I Amin, AM Idris, S Mansoor, ID Bedford, P Dhawan, N Rishi, SS Siwatch and AM Abdel-Salam, 2003. Diversity of DNA beta, a satellite molecule associated with some monopartite begomoviruses. Virology, 312: 106-121.

Briddon RW, SE Bull, I Amin, S Mansoor, ID Bedford, N Rishi, SS Siwatch, Y Zafar, AM Abdel-Salam and PG Markham, 2004. Diversity of DNA 1: a satellite-like molecule associated with monopartite begomovirus-DNA beta complexes. Virology, 324: 462-474.

Bull SE, RW Briddon and PG Markham, 2003. Universal primers for the PCR-mediated amplification of DNA 1: A satellite-like molecule associated with begomovirus-DNA beta complexes. Molecular Biotechnology, 23: 83-86.

Doyle JJ and JL Doyle, 1990. A rapid total DNA preparation procedure for fresh plant tissue. Focus, 12: 13-15.

Fiallo-Olive E, Y Martinez-Zubiaur, E Moriones and J Navas-Castillo, 2012. A novel class of DNA satellites associated with New World begomoviruses. Virology, 426: 1-6.

Guo X and X Zhou, 2005. Molecular characterization of Alternanthera yellow vein virus: a new begomovirus species infecting *Alternanthera philoxeroides*. Journal of Phytopathology, 153: 694-696.

Ha C, S Coombs, P Revill, R Harding, M Vu and J Dale, 2008. Molecular characterization of begomoviruses and DNA satellites from Vietnam: Additional evidence that the New World geminiviruses were present in the Old World prior to continental separation. Journal of General Virology, 89: 312-326.

Haider MS, MTS Latif and RW Briddon, 2005. First report of Tomato leaf curl New Delhi virus infecting Eclipta prostrata in Pakistan. New Disease Report, 11: 39.

Hussain K, M Hussain, S Mansoor and RW Briddon, 2011. Complete nucleotide sequence of a begomovirus and associated betasatellite infecting croton (*Croton bonplandianus*) in Pakistan. Archives of Virology, 156: 1101-1105.

Juarez M, R Tovar, E Fiallo-Olive, MA Aranda, B Gosálvez, P Castillo, E Moriones and J Navas-Castillo, 2013. First Detection of Tomato leaf curl New Delhi virus Infecting Zucchini in Spain. Plant Disease, 98: 857-857.

Mansoor S, RW Briddon, Y Zafar and J Stanley, 2003. Geminivirus disease complexes: An emerging threat. Trends in Plant Science, 8: 128-134.

Mansoor S, Y Zafar and RW Briddon, 2006. Geminivirus disease complexes: the threat is spreading. Trends in Plant Science, 11: 209-212.

Martin D and E Rybicki, 2000. RDP: detection of recombination amongst aligned sequences. Bioinformatics, 16: 562-563.

Mazhar H, S Mansoor, I Shazia, Y Zafar and RW Briddon, 2000. First report of Tomato leaf curl New Delhi virus affecting chilli pepper in Pakistan. New Disease Report, 9: 20.

Mubin M, RW Briddon and S Mansoor, 2009a. Complete nucleotide sequence of chili leaf curl virus and its associated satellites naturally infecting potato in Pakistan. Archives of Virology, 154: 365-368.

Mubin M, RW Briddon and S Mansoor, 2009b. Diverse and recombinant DNA betasatellites are associated with a begomovirus disease complex on Digera arvensis, a weed host. Virus Research, 142: 208-212.

Mubin M, MS Shahid, MN Tahir, RW Briddon and S Mansoor, 2010. Characterization of begomovirus components from a weed suggests that begomoviruses may associate with multiple distinct DNA satellites. Virus Genes, 40: 452-457.

Mubin M, S Akhter, I Amin, Briddon and Mansoor, 2012. *Xanthium strumarium* - a weed host of components of begomovirus-betasatellite complexes affecting crops. Virus Genes, 44: 112-119.

Nawaz-Ul-Rehman MS, N Nahid, S Mansoor, RW Briddon and CM Fauquet, 2010. Post-transcriptional gene silencing suppressor activity of two non-pathogenic alphasatellites associated with a begomovirus. Virology, 405: 300-308.

Nawaz-ul-Rehman MS, RW Briddon and CM Fauquet, 2012. A melting pot of Old World begomoviruses and their satellites infecting a collection of Gossypium species in Pakistan. PLoS One, 7: 8.

Tahir M and MS Haider, 2005. First report of Tomato leaf curl New Delhi virus infecting bitter gourd in Pakistan. Plant Pathology, 54: 807.

Vinoth KR, D Singh, AK Singh and S Chakraborty, 2017. Molecular diversity, recombination and population structure of alphasatellites associated with begomovirus disease complexes. Infection, Genetics and Evolution, 49: 39-47.

Zaidi SS, S Shakir, M Farooq, I Amin and S Mansoor, 2017. First Report of *Alternanthera yellow vein virus* from *Eclipta prostrata* in Pakistan. Plant Disease, 101: 266.