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Research Article

Molecular detection of disease-causing agent infecting *Malvastrum coromandelianum* L. in Bahawalpur District Punjab Pakistan

Ali HASNAIN[1], Hsi-Hsien YANG[2]*, Muhammad Zia-UR-REHMAN[3] Sohaib MOHAMMAD[1], Zahid MEHMOOD[1], Ayesha IQBAL[1] Arooba JOHN[1], Binyameen[1], Maryam IQBAL[1], Naveed ANJUM[1], Iftikhar HUSSAIN[4], Zafar I. KHAN[5] Muhammad U.F. AWAN[1]*

[1]*Government College University, Department of Botany, Lahore 54000, Pakistan; imalichoudhary@gmail.com; dr.shoaibmuhammad@gcu.edu.pk; zahidmehmood9037@gmail.com; ayeshaiqbal706@gmail.com; Aroobajohn5@gmail.com; meen3014@gmail.com; maryamiqbalgcu@gmail.com; mnavid266@gmail.com; dr.umerfarooqawan@gcu.edu.pk (*corresponding authors);*
[2]*Chaoyang University of Technology, Department of Environmental Engineering and Management, Taiwan; hhyang@cyut.edu.tw (*corresponding author)*
[3]*Government Graduate College of Science, Wahdat road Lahore, Pakistan; zmansbs@gmail.com*
[4]*University of Vigo, Department of Plant Biology and Soil Science, 36310-Vigo, Spain; iftikhar@uvigo.es*
[5]*University of Sargodha, Department of Botany, Sargodha, Pakistan; zafar.khan@uos.edu.pk*

Abstract

Weeds are common ancestors of modern crops. They are critical source of genes for resistance to diseases, pests and stresses such as drought and extreme environmental conditions. The wild relatives led to improve resistance to wheat curl mite, late blight in potato and to grassy stunt disease in rice. Agricultural pathogens are infecting economically important cash crops and weeds as well. *Malvastrum coromandelianum* is highly invasive weed specie of family *Malvaceae*. It is distributed all over the world in all climatic condition of tropical, sub-tropical and temperate region. The Malvaceae family is economically important as cotton (*Gossipium hirsutem*) belongs to it. *M. coromandelianum* has ethnobotanical importance and is extensively applied in the treatment of different diseases in south Punjab of Pakistan. It has been observed that there is a specific disease that affects the leaves of *M. coromandelianum*. In this study, we investigated the disease-causing agents (viruses and bacteria) at molecular level. Molecular investigation includes DNA isolation of unknown pathogens, verification through agarose gel electrophoresis, for identification of bacterial pathogens, 16s RNA primers were applied, and for viral pathogen using specific primers and RCA (rolling circle amplification) product as template. PCR was carried out for the amplification of full-length genomes. Manifestation of 1.4 kb bands showed the presence of the alpha and beta satellite of *begomoviruse*. Two types of *begomoviruses*, *Malvestrum yellow vain younnan virus* (MaYVYV) and *Malvastrum yellow vain* virus (MaYVV) was confirmed in this weed. The intermediate host *Bemisia tabaci* is the most common for the *begomovirus* transmission in crops and weeds. Intercrops and trap-crops techniques decrease the rate of whitefly population. Cultural methods, use of chemicals and biocontrol are useful practices to eliminate whitefly from field.

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Keywords: Alpha and beta satellite; Begomoviruse; *Bemisia tabaci*; Geminiviridae; Malvaceae

Introduction

Malvastrum coromandelianum is an invasive weed (Shaiphali *et al*., 2020) of gardens, field, and waste land (Pandey 2016). The plant stem is erect and have lots of hairs. The leaves are alternate simple, and elongated. The flowers are solitary or in small group in terminal position. The flower colour is pale yellow to yellow orange. The fruits are dry, flattened and disc shaped. Stem is green when young but in older it turns brown, covered by hairs. The *M. coromandelianum* is a perennial herb. It acts as a weed in the cotton fields. The seeds are matured within 3 to 4 months. It has been observed that plants contain two types of seeds. Some are large and brown (fertile) and another are small and black (Sterile). The large and brown (fertile) seeds are used for germination study. The seeds of *M. coromandelianum* are greyish brown in colour. The large, viable and fertile seeds are brown and small and sterile seed are black in colour. They act as a reservoir of *begomoviruses*. Its natural habit helps in the transmission of *begomoviruses* in cotton. This weed has many cases of economic importance. Its aerial parts are used for the extraction of essential oil, alkaloids and phenolic contents. Its antibacterial, anti-inflammatory, anti-fungal, anti-diabetic, anti-diarrheal, anti-inflammatory etc. properties make it unique from others (Saxena *et al*., 2020). *M. coromandelianum* is used by various tribes in past for wound healing, fever, wound bleeding, cough, sore throat, hepatitis and for arthritis (Li, 2006). *Malvastrum coromandelianum* water extract has significant inhibition role on carrageenan induced rat hind pawedma (Khonsung *et al*., 2006). A study showed that 80% people in developing countries use herbal products for the treatment of various infection like burn, cut, and wounds. The ethanolic extract is helpful for the cure of wound healing in humans (Gangrade *et al*., 2012). More than 8 million children under the age of 5 are infected with diarrhea (Dosso *et al*., 2012). M*alvastrum tricuspidatum* extract of ethanol 95% inhibit the infection of diarrhea. The extraction through chloroform and methanol from the whole plant body is effective against antifungal and antibacterial activities. Inflammation is system of defence against tissue damage in plants. *M. coromandelianum* has the properties to absorb the heavy metals (zinc, lead, and copper) from soil without damaging the cell of roots and shoots. The concentration of these metals was found in mg/kg in the *M. coromandelianum* body. The *M. coromandelianum* secondary metabolites such as terpenoids, steroids and alkaloids have excellent actinoceptive and analgesic activities with no side effect. The study showed that acetone leaf extract is helpful for destroying the dengue larvae (Yadav *et al*., 2015). The *Geminiviridae* is the plant virus's family. It is the most important family that cause diseases in economically important crops and weeds (Fiallo *et al.,* 2021). The first description about the geminiviruses was given by Goodman in (1977). The genome of *geminiviruses* consists of single-stranded DNA. The geminate particles are associated with *Cassava latent virus* (CLV) that is also known as *Africa cassava mosaic viruses* (ACMV), and *Bean golden mosaic virus* that is also known as *Bean golden yellow mosaic virus* (BGYMV), contained single stranded DNA genomes (Goodman,1977). *Geminiviruses* widely infect the wheat, maize and cassava etc. For gene expression *Gminiviruses* use different overlapping transcript (Navas and Fiallo, 2021). Begomovirus is the biggest genus of the family *Geminiviridae* and have lot of economical properties worldwide. The genus has more than 400 species that were recognized by the International Committee on Taxonomy of Viruses (ICTV) (Adams *et al*., 2017). This virus is transmitted by the whiteflies (*Bemisia tabaci*) (Zerbini *et al*., 2017) and infect the dicotyledonous plant such as cotton, tomato, chilies and Malvaceae family members (Brown *et al*., 2015). The begomovirus replicate in salivary glands of *Bemisia tabaci* that cause yellow leaf curling in tomato (TYLCV) (Hea *et al*., 2020). The genome of monopartite begomovirus consist on single component which is homologous to the DNA-A which is component of bipartite begomoviruses. There are three kinds of satellites

associated with monoparatite, alphasatellite, betasatellite and deltasatellite (Lozano *et al*., 2016). Bipartite begomoviruses genome also contain two types of components, DNA-A and DNA-B. The length of the component is 2.5 to 2.8kb (Fiallo *et al*., 2021). In the component of DNA-A, it encodes the six types of proteins which are involved in encapsidation, viral replication, pathogenesis and transmission (Fondong *et al*., 2013). In the case of DNA-B, the component encodes two type of proteins which take part in systemic spread and cell to cell throughout the host. On the basis of phylogenetic analysis of full length, the begomovirus are classified into Old World and New World Virus (Briddon *et al*., 2018). Old world begomoviruses are those viruses that originate from Asia, Europe, and Australia such as monopartite. The New World begomoviruses originate from South and North America such as Bipartate. There is no evidence about monopartite begomovirus identification from new world. They are identified only in Old World (Zhang and Ling *et al.,* 2011). The whitefly is the major pest that infect and damages the agriculture system. It is the most common vector of many viral infectious diseases. Family *Malvaceae* is known as Mallow family. The family members are very unique and are herbs, shrubs and trees. Cotton, Okra, Papaya, Malvestrum and Hollyhock are some members of family Malvaceae. They are infected by diverse group of begomoviruses. Weeds are the host for plant viruses. Many kinds of weeds have been identified as the alternative hosts of geminiviruses (Fiallo *et al*., 2012). One of them is *M. coromandelianum* which is tolerant to harsh conditions. *M. coromandelianum* is infected by the *begomovirus*.

The main objective of this study is to identify the disease-causing agent at molecular level in *M. coromandelianum* in Bahawalpur District of Punjab, Pakistan. Bahawalpur is the central area of cotton production in the Punjab. It produces about 40% cotton of the Punjab and 30% of Pakistan. Cotton production for Pakistan's textile is lifeline and it is important cash crop. Cotton cultivation starts in the month of April to June, during monsoon season, on 14% the cultivated land area. 1.5 million farmers grow cotton every year in Punjab and Sindh province. But from 2014 the cotton production has been in steep decline. From 2014-2021, 61% decrease was seen in cotton production. Its production has dropped to estimated 4.5 million bales in 2020/2021. The main reason behind this was climatic change and changes in genetics of cotton by viral diseases (Rehman, 2021). Cotton leaf curl disease first time seen in Nigeria in 1912. It spread to other countries including Pakistan, USA, China and India (Rehman *et al.,* 2017). *M. coromandelianum* act as an intermediate host for begomovirus in cotton fields. This virus badly affects the cotton and other crops. The yield of cotton is reducing day by day which cause economical loss to farmers. The intercropping is a useful approach to control the spread of gymniviruses (Fonding *et al.,* 2013). It is process in which we grow other vegetation in the field to protect our desired crops or vegetation from the effect of whiteflies. In the different plant presence, the rate of movement and feeding behaviour of whiteflies become more sporadic. The shorter feedings time slower the rate of transmission of virus. Another approach is crop rotation. This approach is helpful for the breakdown of the life cycle of weeds, insect vectors and different diseases cycles (Rampersad and Umaharan, 2007). Rouging process is also assist in the delaying of spread. The removal of infected material from the field help to minimize the infection of virus and other diseases. Discarding, destroying and uprooting eliminate the dispersal of whiteflies to other plant. The pyriproxyfen and buprofezin are chemicals to kill the whiteflies but the use of chemicals may harm the vegetation, so it is necessary to monitor the vegetation carefully to restrict the damage (Palumbo *et al.,* 2001). Biological control in which we introduce the predators and parasitoids in the field to kill the whiteflies.

Materials and Methods

Collection of plant samples

M. coromandelianum is a weed that shows yellowing of veins and leaf curling symptoms, shown in Figure 1. Leaves were collected from different sites of district Bahawalpur, province Punjab, Pakistan in month of July

2021. The samples were collected, placed in polythene zip bags and labelled with permanent marker according to date and place of collection. Symptoms showing leaves were photographed with high resolution camera. The samples were than stored in -20 °C freezer for further activities.

Figure 1. *Malvestrum coromandelianum* showing yellowing of veins and curling of leaves

Extraction of DNA by CTAB method

Total genomic DNA was extracted from infected leaves of *M. coromandelianum* applying CTAB method of (Kumar *et al*., 2012). The leaf samples were grinded to make a fine powder with the help of pestle and mortar in the presence of CTAB buffer. After grinding the samples were transferred to Eppendorf. 30 µl of extraction buffer was added and mixed gently with the sample. The samples were incubated at 65 °C and (invert the tube after 15 minutes) for 40 minutes. The mixture was allowed to cool for few minutes. Centrifuge the samples at 10000 rpm for 10 minutes. Then the upper most layer was transferred into a fresh Eppendorf. Add equal volume of extraction buffer and keep it again for incubation at 60 °C for 30 minutes. Centrifuge the samples at 12000 rpm for 15 minutes. Transfer the aqueous layer into new Eppendorf. Add double volume of (60µl) chloroform (24:1) and gently invert the tubes 10-15 times and centrifuge again at 12000 rpm for 15 minutes. If the layer is not transparent then repeat the step again until the solution is transparent. Now add double volume of chilled isopropanol (60µl) and keep the Eppendorf overnight in -20 °C freezer. Centrifuge at 10000 rpm for 15 minutes and discard the supernatant layer. Pellets were formed. Pellets were washed with 70% ethanol and dried at 37 °C. Now add the 60 µl TE buffer and add 3 µl of R-nase A into tube. When the process of incubation completed, added 3µl proteinase-K and incubated the Eppendorf at 37 °C for the time period of 10minutes. Dissolved the pellets in TAE buffer by pipetting and store at -20 °C for further procedure.

Agarose Gel Electrophoresis

Agarose gel electrophoresis applied to check the quality and size of DNA (Lee *et al*., 2012) by using following method: 1% of agarose was made in TAE buffer. 5µL of ethidium bromide was added in the gel and poured in caster containing combs for wells formation. After solidification of gel caster was placed in the electrophoretic tank and combs were removed. DNA samples were loaded in the wells using loading dye. Voltage was supplied 90 V for 30 minutes presence of DNA was checked in trans- illuminator.

DNA Quantification

The DNA quantification was done by double beam spectrophotometer. Spectrophotometer determined the concentration of DNA by taking the absorbance at 260nm / 280nm. The formula use for the calculation of DNA concentration is given below (Morris, 2015).

DNA µg/mL = E (50) × OD260nm × Dilution factor

E is the values of extinction coefficient.
OD is optical density of DNA.

Polymerase chain reaction for bacterial detection

16rsRNA universal primers are used to amplify the bacterial DNA with following sequence describe by (Carl and George in 1977).27 Forward: 5'-AGAGTTTGATCCTGGCTCAG-3', 1492 Reverse: 5'-GGTTACCTTGTTACGACTT-3'.

Reaction mixture for PCR

The composition of reaction mixture was according to this, PCR Master Mix: 12µL, DNA: 2µL, Forward Primers: 0.5µL, Reverse Primers: 0.5µL, dd. water 10µL, Total: 25µL.

PCR profile

PCR profile was optimized according to the following condition, Initial Denaturation 94 °C 30 seconds, Denaturation is at 94 °C for 3 minutes, Annealing is at 57 °C for 30 seconds, Extension at 72 °C 60 seconds, Elongation is 72 °C for 5 minutes, Total 30 cycles.

Polymerase Chain Reaction for virus detection

The basic principle of PCR (Polymerase Chain Reaction) is the amplification of the Cp-core regions in DNA by using Cp-core related primers (Sambrook and Russell, 2001). The composition of reaction mixture was $Mgcl_2$: 1.5µL, DNA: 1µL, 10X TAQ buffer: 2.5Ml, cp-Core F-primer: 1µL, cp-Core R-primer: 1µL, 10mMdNTPS: 2.5 taq polymerase: 0.25µL dd. H2O: 15.25µL, Total = 25µL.

PCR Profile

PCR profile was optimized according to the following condition, Initial denaturation is at 95 °C for 5 minutes, Denaturation is at 95 °C for 1 minute, Annealing is at 52 °C for 1 minute, Extension at 72 °C 1 minute, Elongation is 72 °C for 10 minutes, Total 35 cycles.

Rolling circle amplification

A short primer of RNA or DNA is amplified by using an isothermal enzymatic process called RCA. By using a circular DNA template or polymerase we can form a long single stranded DNA or RNA. The DNA was amplified by RCA according to following conditions, DNA template; 4Ml, Random primers; 3µL, dNTPs; 3Ml, dd. Water; 14.5Ml, Pyrophosphate:1.5Ml, ϕ 29 buffer =3µL, ϕ 29 = 1µL, Total Volume; 30µL. Incubate at 65 °C for 10 minutes. Gel electrophoresis was done to check the exact band of DNA. Incubate at 65 °C for 10 minutes. Gel electrophoresis was done to check the exact band of DNA.

PCR for amplification of virus and associated satellites (Betasatellite & Alphasatellite)

Polymerase chain reaction (PCR) was carried out to amplify the full-length genomes of *begomoviruses* and associated satellite (betasatellite and alphasatellite) region in DNA samples by using their specific primers (Zia-Ur-Rehman *et al*., 2013). Formulation of PCR reaction mixture for detection of beta-satellite and alpha-satellite, Master mix:15µL, dd. Water:13µL, beta F-primers:1µL, beta R-primers:1µL, total volume will be 30 µL.

Primer region for Begomovirus

These are the pairs of primers that is described by Zia-Ur-Rehman *et al*. (2013) were used for the isolation of full-length genome of *begomovirus*.

Begomovirus F =5′-ACGCGTGCCGTGCTGCTGCCCCCATTGTCC-3′,
Begomovirus R =5′-ACGCGTATGGGCTGTCGAAGTTCAGAC-3′

Primer region for Betsatellite

Following pairs of primers were used for the isolation of begomovirus associated betasatellite as described in (Zia-Ur-Rehman *et al*., 2013).

Beta F = 5′-GGTACCGGAGCTTAGCWCKCC-3′,

Beta R = 5′-GGTACCGTAGCTAAGGCTGCTGCG-3′

Primer region for Alphasatellite

Following pairs of primers were used for the isolation of begomovirus associated alphasatellite as described in (Zia-Ur-Rehman *et al.,* 2013). Alpha F = 5′-AAGCTTAGAGGAAACTAGGGTTTC-3′, Alpha R = 5′-AAGCTTTTCATACARTARTCNCRDG-3′

PCR Profile

PCR profile was optimized according to the following condition: initial denaturation is at 95 °C for 5 minutes, denaturation is at 95 °C for 1 minute, annealing is at 52 °C for 1 minute, extension at 72 °C 1 minute, elongation is 72 °C for 10 minutes, Total 35 cycles from step 2-4.

Results

Total genomic DNA Isolation and Quantification

The genomic DNA was extracted from infected leaves. Results were checked by running the samples on 1% agarose gel. 5 samples out of 7 had a good quality of DNA with minor differences in final concentration as shown in Figure 2.

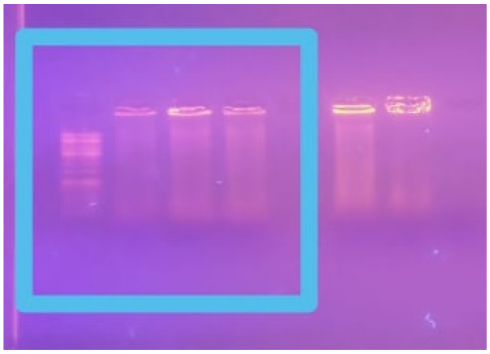

Figure 2. 1% Agarose gel electrophoresis showing genomic DNA of samples. L is ladder. S1, S2, S3, S4, S5 shows the band of genomic DNA

DNA Quantification

DNA quantification was done by using the UV/VIS spectroscopy at the absorbance of 260 and 280 nm respectively. The results are as shown in Table 1.

Table 1. Result of DNA quantification

Samples	DNA Concentration ng/µL	Absorbance at 260 nm	Absorbance at 280 nm	Absorbance ratio 260/280 nm
S1	300.12	2.72	1.47	1.85
S2	298.94	2.65	1.48	1.79
S3	305.44	2.83	1.53	1.84
S4	287.1	2.45	1.35	1.74
S5	293.67	2.35	1.30	1.80

PCR for bacterial detection

16srRNA used for the bacterial detections on gel. 7 wells are showing the bacterial DNA. The result of gel is showing in Figure 3.

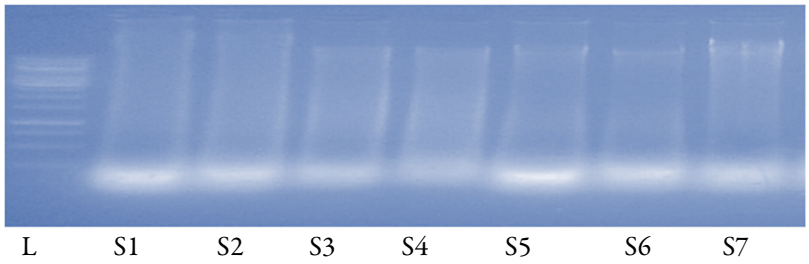

Figure 3. 1% agarose gel showed the PCR result of 16srRNA primers

PCR for virus detection

PCR was carried out and the presence of *begomovirus* was checked with the help of specific CP core region for forward and reverse primers (Zia-Ur-Rehman *et al*., 2013). Two samples showed the presence of *begomovirus*. Amplified fragments were observed at 600bp by running on gel (Figure 4).

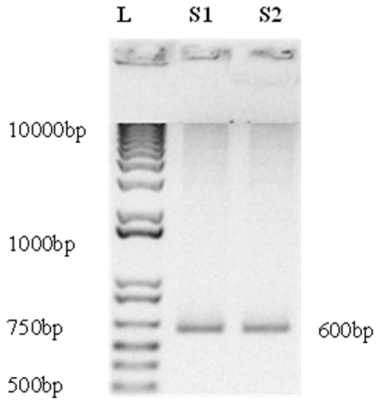

Figure 4. Agarose gel electrophoresis showing the result for Cp-core amplified PCR product, L is ladder and S1 and S2 are samples

Rolling circle amplification

RCA was used for the amplification of DNA fragment by using Φ29 polymerase. RCA band was observed on 1% agarose gel as shown in Figure 5.

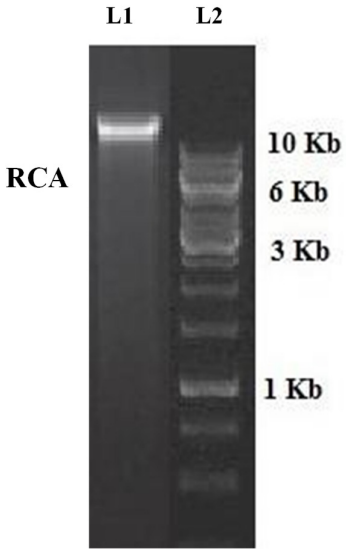

Figure 5. 1% agarose gel showing RCA product results

Amplification of Alpha and Betasatellite

PCR reaction was carried out to amplify the Alphasatellite and Betasatellite from sample. Manifestation of 1.4kb bands in the results was a clear description of the presence of the alpha and beta satellite (Figure 6).

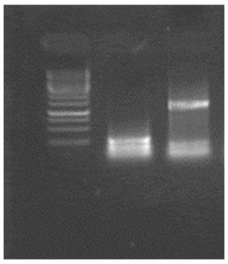

Figure 6. Agarose gel electrophoresis showing the restriction digestion of alpha satellite and beta satellite. Lane 1 contains DNA ladder, lane 2 and 3 contains amplified band

Discussion

In Asia, economically important diseases were mostly recorded in the Indian subcontinent. Indian cassava mosaic virus, *Mung-bean yellow mosaic virus*, *Tobacco leaf curl virus*, *Yellow vein younan virus* and *Cotton leaf curl virus* are among the most common geminiviruses (Roberts, 1989). *M. coromandelianum* belong to the family *Malvaceae* which has been used for medicinal purposes. The plants of this family are well known for their antibacterial and antifungal activities due to the presence of alkaloids and essential oils (Sittiwet *et al*., 2008). They also act as a weed. Weeds can survive easily in cropping and non-cropping season in or around the field. They make the weeds reservoir of many different plant viruses (Wang *et al.,* 2021). *Malvastrum coromandelianum* is a widespread weed in Pakistan and reported host of *Holyhock leaf curl virus* and *yellow vein younnan virus* in Pakistan (Zia-Ur-Rehman *et al.,* 2018). This weed plays an important role in the transmission of *begomovirus* into the economicaly important crops with the help of insects (whiteflies) as shown in Figure 7.

Figure 7. Cotton leaves are affected by whitefly and CLCuV (*Begomovirus*)

This transmission causes the damage of important crops. After DNA extraction presence of two species of begomovirus is confirmed. In our study we identified the *Malvestrum yellow vain younnan virus* (MaYVYV) and *Malvastrum yellow vain* virus (MaYVV). These viruses produce yellow vain and mosaic symptoms in *Malvastrum corromandelianum*. The occurrence of the whole complex consisting of monopartite virus along with alphasatellite and a beta satellite in a weed species clearly indicates the expanding host range of begomoviruses. We can control the transmission of the *begomovirus* through whiteflies by using different old cultural methods, chemicals and by bicontrols.

Conclusions

The present study highlighted that there is a need to collect more and more systematic data about the alternate host of cotton infecting viruses. The use of sentinel plots in which growing all possible hosts of the *begomoviruses* along with cotton in the same location and also look for the alternate hosts which serve as a reservoir of these viruses in winter season, when cotton is not growing in the field. After the complete data of virus population in crops an infective strategy can be apply to combat the problem. Many crops and weeds are infected and destroyed due to viral infection. The annual production of crops decreases day by day. The economy of the country is also damaged due to viral diseases. Pakistan is an agricultural land. The province Punjab and Sindh of Pakistan has fertile soil for agriculture. Most of the production is from these two provinces. In the present study it is confirmed that the two types of *begomovirus*, *Malvastrum yellow vain yonnan virus* (MaYVYV) and *Malvastrum yellow vain virus* (MaYVV) are infecting the *Malvastrum corromandelianum*. The presence of begomoviruses in a single host causes exchange of genetic material by recombination. Through this recombination new viral diseases are start emerging in the field. This weed plays the role of secondary host for the transmission of virus to other economical important crops such as in cotton, okra, tomato, chilies etc. This weed can easily grow in the field. The use of chemicals and biological control for whiteflies (primary host) is very important to eliminate the viral causing agent from the field.

Authors' Contributions

AH collected the samples. MZUR helped in identification of disease showing symptoms of plant. Sohaib Mohammad helped in arranging data in tables. AH, AI, ZM, AJ, B, MI, and HNA contribute in molecular studies and wrote the Research paper. Research work was done under the supervision of MUFA. IH, ZIK analyzed the research paper and identified some mistakes, and solved out. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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