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RESEARCH ARTICLE

Molecular Characterization of Pathogen, Infecting *Capsicum Frutescene* (Chilli Papper) And *Solanum Lycopersicum* (Tomato) In Lahore, Pakistan

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A B S T R A C T

Chili pepper (*Capsicum frutescens* L.) and tomato (*Solanum lycopersicum* L.) are the herbaceous plants that are cultivated all over the world. In recent years, the production of chili peppers and tomatoes has been hampered by several diseases. This study was carried out for molecular characterization of pathogens infecting *Capsicum frutescens* L. and *Solanum lycopersicum* L. The Leaves with disease-associated symptoms like leaf spots, curling, wilting, and yellowing of tips and veins, were collected from vicinity of the Lahore city. For molecular characterization, DNA was isolated from infected leaves through CTAB method. Internal Transcribed Spacer primers (ITS1-F, ITS4-R) were used for identification of fungal pathogens and Primers targeting the viral cp-core region were applied to find viral pathogens. Rolling Circle Amplification (RCA) was completed using random primers to amplify circular viral genomes. The amplicon of Polymerase chain reaction (PCR) of 600bp was observed on 1% agarose gel electrophoresis representing geminivirus. Furthermore, *geminivirus*-associated betasatellites of 1.4 kb and 2.8 kb genome confirmed the attack of its genus begomovirus in chili and tomato plants. Use of chemicals, screening (symptomatic or asymptomatic), cultural methods, and biocontrol are significant practices to eliminate whiteflies (*Bemisia tabaci*- host of begomovirus transmission) from the field, ultimately it will reduce the infection rate, and increase the production of pathogens-free chili peppers and tomatoes.

Keywords: Begomovirus, Fungal Pathogens, Solanaceae, Whitefly, ITS, cp Core primers.

INTRODUCTION

Solanaceae is a family of flowering plants (Gebhardt, 2016) (*Solanum lycopersicum* L.) tomato and Chili (*Capsicum frutescens* L.) are herbaceous fruiting plants of this family. They are grown in tropical regions and widely eatable all over the world (Carurso *et al.,* 2022). Tomatoes and chilies are the main ingredients of our daily meals. They are rich in vitamins, minerals and various phytochemicals with high medicinal values (Gerszberg *et al.,* 2015; Arballo *et al.,* 2021; Naureen *et al.,* 2022; Azlan *et al.,* 2022; Bjarnadottir, 2023). Fungi, bacteria and viral pathogens cause damage to cash crops and serious losses to the economy worldwide (Singh, 2014). Plant pathogens significantly reduce the quality and

quantity of cash crops. These losses pose a major threat to global food production annually (O'Brien, 2017). The climatic conditions including humidity, rainfall and high temperature, in tropical and sub-tropical regions, contribute to the pathogens burden and mycotoxin contamination in pepper and tomato crops. In recent years the members of the family *Solanaceae* have been greatly infected by fungal and viral pathogens (Hancinsky *et al.,* 2020). Disease symptoms are apparent during flowering and fruiting stages, the plants typically show severe symptoms on leaves (Worku and Sahe, 2018). Comparatively fungal pathogens cause more destructions than other pathogens (Hussain and Usman, 2019). Fungi cause diseases in tomato and chili plants (Kim *et al.,* 2007). Fungal and viral pathogens show similar symptoms in tomatoes and chili peppers that confuse one another (Hussain and Abid, 2011; Atiq *et al.,* 2022).

Gray mold, gray leaf spots, and defoliation are prominent symptoms that cause the reductions in size and number of fruits in chili (Karbowy-Thongbai and Gotz, 2023). Fungus also causes wilting of leaves as a result, leaves turn yellow and then die in both tomato and chili (Sandani and Weerahewa, 2018; Thambugala *et al.,* 2020). Severely infected leaves turn yellow and senesce from the plant of chili pepper. The tomato and chili pepper plants that are infected by viruses, show leaf spots, curling, stunted crinkled, yellowing of veins and tips, and have small fruits or no fruits (Atshan *et al.,* 2018).

Infected plants also show dieback symptoms before flowering (Kwak *et al.,* 2022). Previous studies revealed that in economically important crops, the family *Geminiviridae* is causing diseases (Fiallo-Olivé *et al.,* 2021). Begomovirus is its biggest genus which has been identified from *cucurbitaceous*, *malvaceous,* and *solanaceous,* vegetables in Southeast Asia (Brown *et al.,* 2015). *Bemisia tabaci* (whitefly) is the host of begomovirus transmission (Atiq *et al.,* 2023) and most common vector of many viral diseases (Zerbini *et al.,* 2017). Alphasatellite, betasatellite, and delta satellite are associated satellites of monopartite begomovirus (Lozano *et al.,* 2016).

The family *geminiviridae* was first time established in 1978 by Goodman, (1981) and it consists of 9 genera Becurtovirus*, Begomovirus* (Bipartite genome- DNA-A & DNA-B), *Eragrovirus, Curtovirus, Grablovirus, Topocuvirus, Capulavirus, Mastrevirus, and Turncurtovirus* (Monopartite genome- DNA-A) and (Varsani *et al.,* 2017). The *geminiviridae* has more than 400 species, out of 75 are infecting the chili but only 37 viral species infecting the chili are known to the International Committee on Taxonomy of Viruses (ICTV) (Adams *et al.,* 2017; Thomas *et al.,* 2021). *Chili leaf curl disease* (*ChiLCD*) and *Tomato yellow leaf curl virus* (*TYLCV*) are severe diseases which are caused by begomovirus and affecting chili and tomato production in the subcontinent (Thakur *et al.* 2018; Roy *et al.,* 2019). Mixed infections of *Chili leaf curl virus* (*ChiLCV*), *Cotton leaf curl Multan virus* (*CLCuMuV*), and *Tomato yellow leaf curl virus* (*TYLCV*) were also reported in chili (Mishra *et al.,* 2020).

Some other factors that affect production loss are shortage of improved and good varieties, high temperature, weather circumstances and water availability etc. (Chand *et al.,* 2021).

Pakistan is an agricultural land (Rehman *et al.,* 2015; Iqbal *et al.,* 2022) and Punjab province produces a huge production of tomatoes (Ali *et al.,* 2017) and chili peppers (after Province Sindh) (ASF, 2014) which take a part in boosting the economy of the country. According to the Economic Survey, 2022-23 report, the production of chili (48.9%) and tomato (41.7%) greatly decreased as compared to the report of 2021-2022 (http://www.finance.gov.pk). Lahore is the 2nd largest and capital city of Punjab Provenance of Pakistan. According to the report of Agriculture Marketing Wing Punjab, the production of chilies (2021-2022) is 1943kg/hectare in the Lahore division (http://www.amis.pk). On the other hand, the tomato production in the Lahore division is approximately 512kg/hectare (http://www.mnfsr.gov.pk). The main objective of this research work is to characterize the pathogens that are infecting the tomatoes and chili peppers through pathogens-related specific molecular markers.

MATERIAL AND METHODS

Collection of plant samples: Infected tomato and chili pepper leaf samples showing symptoms like yellow mosaics, stunting leaves, yellowing of veins, and leaf curling as shown in figure 1. were collected randomly in vicinities of Lahore city including, Manga Mandi, ShahPur, Maraka, Mohalanwal, and Chungi Amer Sadhu where chilies and tomatoes are grown. 20 isolates of each selected plant leaves collected for pathogenic identification studies. Leaf samples were taken in pre-labeled polythene zip bags. 0.1% mercuric chloride ($HgCl_2$) was used to surface-sterilize of leaves for 1 min. The leaves sample were then rinsed twice with distilled water and dried (Sharma *et al.,* 2005)

The infected leaf samples were placed at -20°C for further analysis.

Figure 1. (a) Pathogen-infected leaves of tomato, (b) Infected leaves of chili pepper.

Media preparation for fungal culturing: To detect the fungal pathogens the infected leaves were cultured followed by Skyes and Rankin, (2014). Two types of fungal media, PDA (potato dextrose agar) and MEA (malt extract agar) were used for the detection of diverse range of fungal pathogens on the basis of their growth rate and nutrition (Black, 2020). Infected leaf samples were washed with distilled water and 2–3 bites of 0.5 cm the leaves were transferred on both fungal media and then incubated at 28^0C (ESCO Isotherm low-temperature incubator IFC110-8).

Extraction of DNA: After the collection of infected samples of leaves total DNA was extracted by CTAB method with minor modifications (Kumar *et al.,* 2012).

Gel electrophoresis: Extracted DNA samples were run on 1% agarose gel to confirm the presence of DNA as described by the method of Lee *et al.,* (2012).

DNA Quantification: The quantity of extracted DNA from infected leaves samples were checked with the help of the double-beam spectrophotometer model Halo BD20 by formula of calculation given by Morris, (2015).

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

E represented the extinction coefficient and on the other hand, OD is the optical density of DNA which was measured as a 260nm spectrophotometer because 260 and 280 nm is the ratio of absorbance to assess the purity of DNA as described the Glasel, (1995).

PCR profile and primers for fungal genome detection: For the amplification of the fungal pathogens Internal Transcribed Spacer (ITS) region primers, were used as described by White *et al.,* (1990), and the detection of amplified products were confirmed through the gel electrophoresis.

ITS1-F 5’-GCTGCGTTCTTCATCGATGC-3’
ITS4-R 5’-TCCTCCGCTTATTGATATGC-3’

PCR for coat protein-core region for virus: There are many methods available for the detection of plant viruses which could be classified into protein-based methods or nucleic acid-based methods. Cp-core region primers PCR is used for the detection of plant viruses and its primers were primarily designed on coat protein and replicase genes (Saiki *et al.,* 1988). PCR was carried out to amplify the coat protein regions in extracted DNA samples by using the coat protein-core primers (Sambrook and Russell, 2001).

F=5'- ATGGCTAGCTTGTGCAAGTGG-3'
R=5'- TGGAGTCTGCGGGATTCACG-3'

Rolling Circle Amplification profile: For the amplification of a short primer of DNA of plant virus family such as geminiviruses, an isothermal enzymatic (Φ29 polymerase) process called RCA (Rolling Circle Amplification) was used (Haible *et al.,* 2006). Results were observed on 1 % agarose gel.

PCR and primers for amplification of associated satellite (alpha & beta): Zia-Ur-Rehman *et al.,* (2013) described the primers for the associated satellite (alpha and beta) for particularly begomoviruses genus of family geminiviruses (Yang *et al.,* 2019).

Beta-F = 5'-GGTACCGGAGCTTAGCWCKCC-3'
Beta-R = 5'-GGTACCGTAGCTAAGGCTGCTGCG-3'
Alpha-F = 5'-AAGCTTAGAGGAAACTAGGGTTTC-3'
Alpha-R = 5'-AAGCTTTTCATACARTARTCNCRDG-3'

Primers for amplification of the Begomovirus genome: The pairs of primers as described by Zia-Ur-Rehman *et al.,* (2013) is used for the detection of genome of begomoviruses. Afterward, gel electrophoresis was done to confirm the PCR product.

F =5'- ACGCGTGCCGTGCTGCTGCCCCCATTGTCC-3'
R= 5'-ACGCGTATGGGCTGTCGAAGTTCAGAC-3'

RESULTS

Fungal culturing: No colonies of fungus were found on both media. This indicated that the tomato and chili pepper were infected by nonfungal pathogens, further confirmation was made through molecular characterization.

Total DNA extraction: Total DNA was observed on 1% agarose gel. All the 10 samples had good quality of DNA ,T1-T5 represents DNA from infected tomato leaf samples and C1 to C5 are DNA from chili samples (Figure 2).

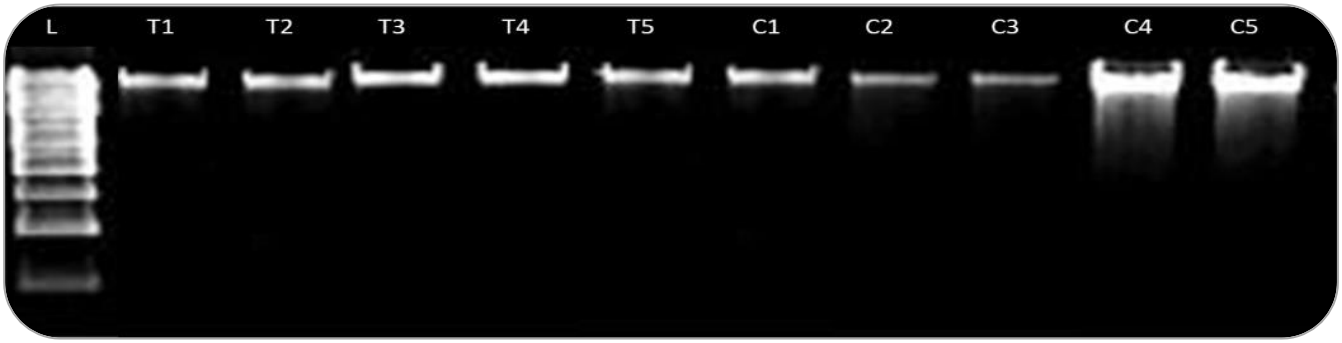

Figure 2. Genomic DNA on 1% agarose gel (L is a ladder, T_1–T_5 are tomato samples, and C_1–C_5 are chili samples)

DNA Quantification: UV/VIS spectroscopy was used for DNA quantification. The mean ratio at an absorbance of 260 and 280 nm were measured (Table 1).

Table 1. DNA concentration and the mean ratio of chili pepper and tomato samples

Sample	Mean ratio (260/280)
T_1	1.75
T_2	1.93
T_3	1.88
T_4	1.79
T_5	1.99
C_1	1.77
C_2	1.82
C_3	1.99
C_4	1.85
C_5	1.71

PCR for detection of coat protein core region of virus: For the successful detection of highly conserved region plant viruses (*geminiviridae*) polymerase chain reaction (PCR) was performed by the specific Cp-core region primers. Amplified fragments associated with cp core region primers conserved region showed 600bp. Result of 600bp on 1% agarose gel confirmed the presence of geminiviruses (Figure 3).

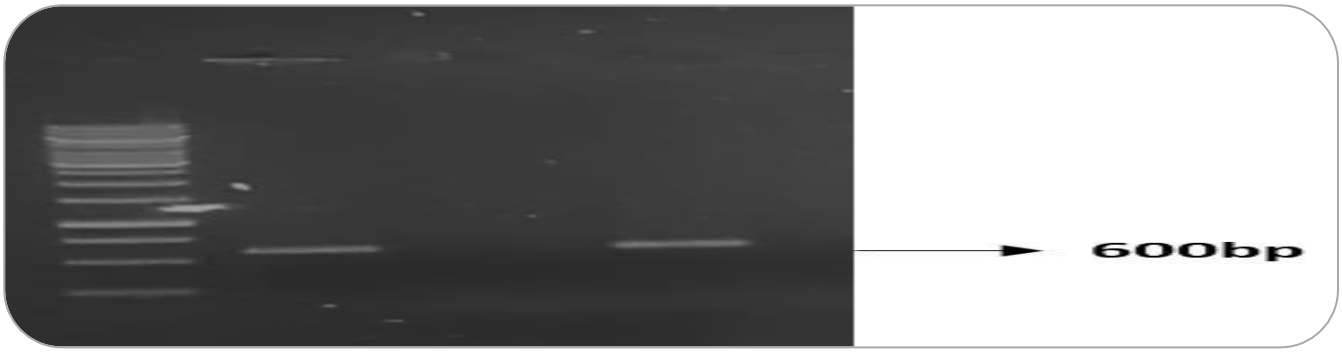

Figure 3. The 1% gel results show Cp-core amplified PCR product

Rolling circle Amplification: Rolling circle amplification was used to amplify the short primer of geminivirus DNA by using Φ29 polymerase. RCA result on the gel of two samples was observed on 1% agarose (Figure 4).

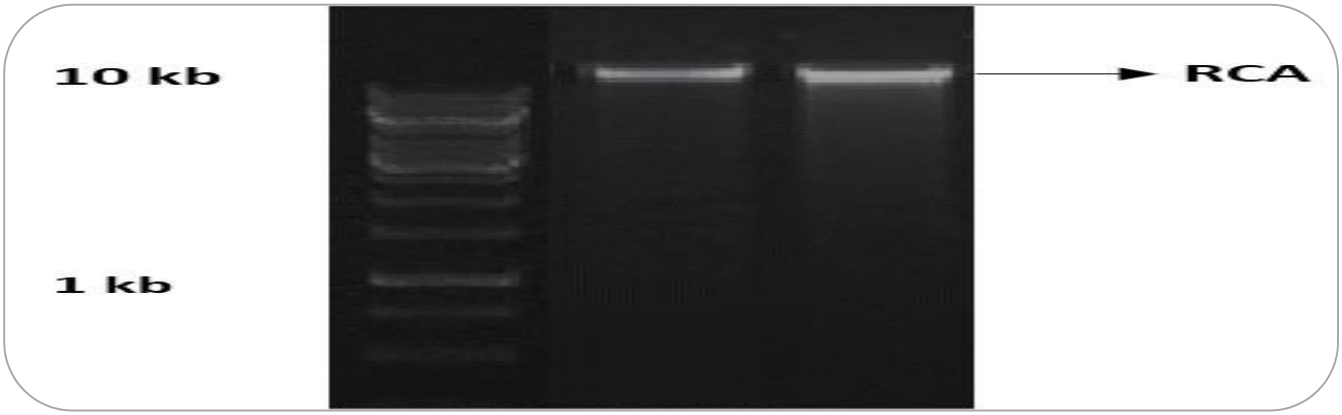

Figure 4. 1% agarose shows the RCA product samples from Lahore

PCR for amplification of associated satellite (alpha & beta) of viruses: PCR was carried out to amplify the associated satellite (alpha & beta). The result of the manifestation of 1.4kb bands on the gel (Figure 5) represents the presence of the associated satellite of (alpha & beta).

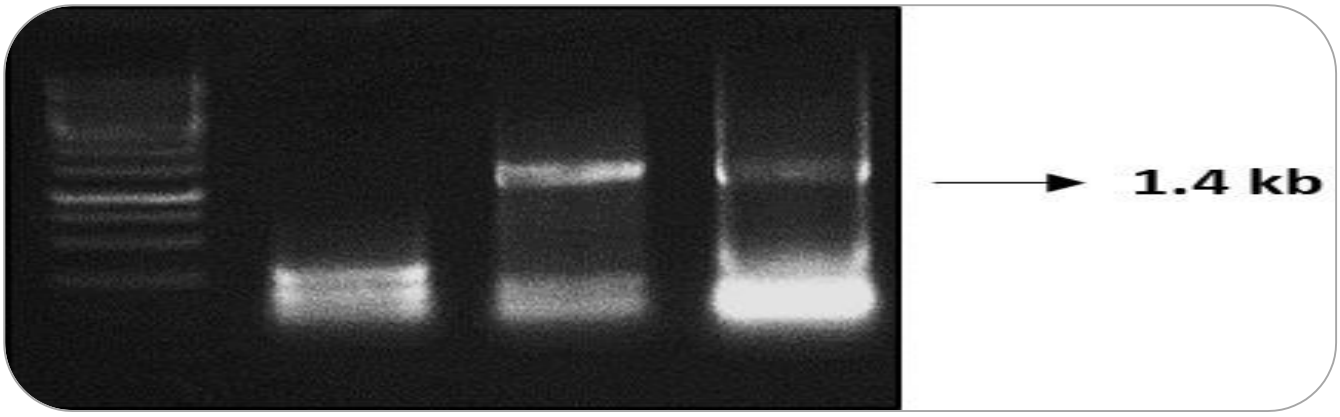

Figure 5. PCR primer result amplified band of 1.4 kb of associated satellite of begomovirus

PCR for Amplification of Begomovirus: The size 2.8kb on 1% agarose gel confirmed the presence of genus begomovirus of family *geminiviridae* (Figure 6).

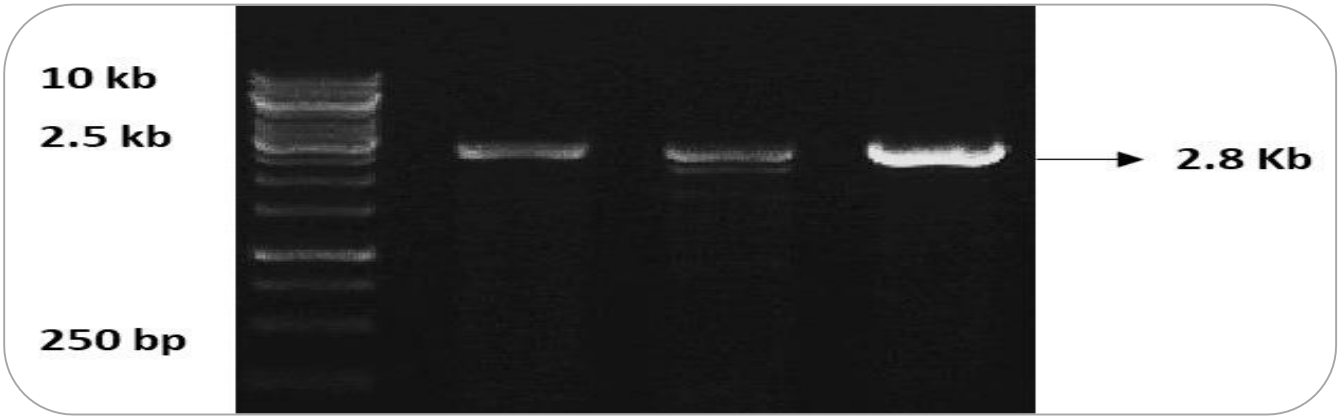

Figure 6. 1% agarose gel shows the amplification of 2.8Kb product of present samples

DISCUSSION

The history of agriculture is associated with biological invasions (Guillemaud *et al.,* 2011). Agriculture sector is challenged by the introduction of pathogens from the global transport of plants and plant products (Jeger *et al.,* 2021). Tomato and chili pepper plants are major vegetable crops in the world (Kwak *et al.,* 2022). According to National Agricultural Research Centre (NARC), the production of tomatoes is around 529,600 tonnes annually and the average yield is 9.2 tonnes per hectare in Pakistan. Around 150,000 acres of farms produce 143,000 tonnes of chili annually and Pakistan falls on number fourth in all over the world for their production (Hassan, 2022). The *Solanaceae* family is majorly affected by pathogens but mostly by genus begomoviruses of family *Geminiviridae* (Varma and Malathi, 2003).

In the present study, detection of pathogens from leaves by PCR-based method was made by using specific primers for specific pathogens (Shad *et al.,* 2023; 24). Primer selection was achieved due to the sensitive and specific detection of pathogens while at the same time minimizing the interference from non-target sequences (Ahmad *et al.,* 2024). Specific amplification with specific primers of DNA was directly completed from infected leaf samples (Machenahalli *et al.,* 2014). PCR technique is an early detection and diagnosis system for many diseases which are infecting chili and tomatoes (Imjit *et al.,* 2013). The present study provides proof that chili peppers and tomatoes are infected by a complex of begomovirus in the Lahore region of Pakistan (Tahir *et al.,* 2023). DNA with 600 bp was extracted which confirmed the presence of family geminivirus. Hence for confirmation of viral pathogenic attack, the most common target was the begomoviral coat protein (CP) gene, and hence specific cp-primers were utilized in this study (Brown *et al.,* 2001). PCR result showed the manifestation of 1.4 kb bands (Figure 5) which confirmed the associated betasatellite of geminivirus. Rolling circle amplification amplified the short primers of geminivirus (Packialakshmi *et al.,* 2010). Amplification of 2.8kb fragment with selected primers confirmed the presence of begomoviruses in chili and tomato plants. The result of 2.8kb on gel (Figure 6) indicated the same finding of Shuja and Tahir, (2019). The findings of (Kwak *et al.,* 2022) also confirmed our results. Further phylogenetic studies and dendrograms are required to characterize the species of begomovirus which infect the tomato and chili pepper (Usman *et al.,* 2024). Begomovirus is expressed as a superior threat that reduces the yield of vegetables worldwide (Leke *et al.,* 2015) and in Pakistan (Hasnain *et al.,* 2022). Studies revealed that in Pakistan, the Punjab province showed a wide diversity of begomoviruses infections in endemic, non-cultivated and cultivated plants (Hussain *et al.,* 2011; Islam *et al.,* 2018; Hasnain *et al.,* 2022). Yellow veins and curling of leaves commonly observed in chili and tomato plants in Pakistan which are caused by begomoviruses (Akhter *et al.,* 2009; Arooj *et al.,* 2017; Yasmin *et al.,* 2018).

Over the most recent couple of years, the high frequency of family *geminiviridae* genus begomovirus species that caused diseases in many important crops and vegetables has been accounted in Pakistan, India, Myanmar and Sri lanka (Sharama *et al.,* 2009; Khan *et al.,* 2014; Shingote *et al.,* 2022). Whiteflies are universal vectors of many important viral infections in tropical and sub-tropical areas (Moodley *et al.,* 2019). Whiteflies transfer both genome (monopartite and bipartite) types of geminiviruses (Brown *et al.,* 2015). In the salivary glands of whiteflies, begomovirus replicates (He *et al.,* 2020). Ten different types of begomovirus species are connected with *Tomato yellow leaf curl virus* (Brown *et al.,* 2015). *Tomato yellow leaf curl virus* (TYLCV) causes 100% production losses, flower abscission, and an overall reduction in normal growth (Diaz-Pendon *et al.,* 2010; Navas-Castillo *et al.,* 2011). The begomovirus can significantly reduce the yield and quality of tomatoes and chili peppers. It affects both local and global trade of these vegetable crops. The usage of eco-friendly pesticides, destruction of infected plants, introduction of resistant varieties and crop rotation etc. can reduce the attack of begomovirus. Addressing these challenges will ensure agricultural sustainability.

CONCLUSION

Pakistan is an agricultural state and Punjab province produces huge production of tomato and chili pepper which take part in boosting the economy of the country. The annual production of chili peppers and tomatoes decreases day by day. This decrease in annual production damages the economy of the country. The present study confirmed that the begomovirus is infecting the economically important vegetable crops i.e. chili pepper and tomato. Future research in this area and the development of targeted control

strategies for specific pathogens can help in the reduction of damage. The resistant varieties should be introduced, controlled atmosphere, crop rotation, heat treatments, biological control agents, horticultural practices, usage of pesticides for whitefly control, and screening of seedlings are some useful practices that can increase production of these crops.

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