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CURRENT STATUS AND MOLECULAR CHARACTERIZATION OF *ZUCCHINI YELLOW MOSAIC VIRUS* (ZYMV) INFECTING RIDGE GOURD (*LUFFA ACUTANGULA* L) IN DIFFERENT REGIONS OF PUNJAB, PAKISTAN

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Abstract

Zucchini yellow mosaic (ZYMV) included in the genus Potyvirus is one of the most destructive pathogens of cucurbits, including ridge gourd (*Luffa acutangula* L.) which is widely grown in Indo-Pak subcontinent and can cause significant yield losses worldwide. In the present study, a total of 300 leaf and fruit samples of ridge gourd with virus-like symptoms were collected from 03 districts of Punjab, Pakistan. To perform an initial screening of ZYMV these samples were subjected to Plate Trap Antigen-Enzyme Linked Immunosorbent Assay (PTA-ELISA) using monoclonal antibodies. Overall disease incidence during 2018-2019 was 28.33%. The prevalence of ZYMV was confirmed in all ridge gourd sampling sites. ELISA-positive samples were further confirmed through RT-PCR and sequence analysis. Comparison of sequences with those available in Genbank showed 91-98% nucleotide and 98%-100% amino acid-based homology. Phylogenetic tree analysis revealed that Pakistani ZYMV ridge gourd isolates (MN897100, and MN897101) have close relationship with South Korean, Chinese and Turkish ZYMV isolates, and strengthened the belief that ZYMV Pakistani isolates reported in this study has Asian origin. Identification of new ZYMV isolates strengthen the breeding programs for the development of resistance genotypes to manage this notorious virus.

Key words: Ridge gourd, ZYMV, PTA-ELISA, RT-PCR, GenBank.

Introduction

The *Cucurbitaceae* family occupied a prominent position among horticultural crops (Adetula & Denton, 2003; Okonmah, 2011). This family nearly comprises of 118 genera and 825 species (Bai, Zhang *et al*., 2016). The members of this family are Cucumber (*Cucumis sativa*), Round gourd (*Lagenaria siceraria),* Bitter gourd (*Momordica charantia*), Ridge gourd (*L. acutangula),* watermelon (*Citrullus lanatus*), and Musk melon (*Cucumis melo*) which are mostly grown in all over the world as well as in Pakistan for the edible purpose (Ali *et al*., 2014). Among all these members Ridge gourd (genus *Luffa*) are grown by all types of farmers, i.e. commercial to marginal in Pakistan because of low input cost compared to other cucurbits. Ridge gourd (2n=26) also famous as Chinese okra, ribbed gourd, angled loofah (*L. acutangula* Roxb.), and locally it is known as Tauri. It is considered as one of the important summer and winter vegetables of South-east Asia enriched in vitamins, minerals and antioxidants. (Karmakar *et al*., 2013). It has many beneficial effects on human health such as blood cleaner, laxative, anti-hepatotoxicity, anti-inflammatory, anti-diabetic and anti-biotic (Asif *et al*., 2017). Additionally, it also plays a vital role in rapid weight loss. Ridge gourd immunize the human body by enhancing digestion activities (Kandoliya *et al*., 2016; Barik *et al*., 2018) Ridge gourd was grown in the area of 2008 ha with 17301 tons of annual production (Anon., 2018-19), which is approximately 8 tones/ ha that is considered as a low yield per hectare in comparison to other vegetables-growing regions of the world. Major constraints of low production are the lack of awareness of farmer regarding the adaptability of feasible cultural practices, unavailability of resistant vegetable genotypes, use of uncertified seed and different biotic, and abiotic factors (Woolhouse *et al*., 2005; Jone, 2009). Among the biotic factors, approximately 35 different viruses have been identified from cucurbits in several countries of the world (Provvidenti, 1996). Viruses transmitted by aphid's vector may cause yield losses up to 100% (Ullman *et al*., 1991). Among all groups of viruses infecting cucurbits the viruses belongs to genus *Potyvirus* i.e. *Zucchini yellow mosaic virus* (ZYMV), *Papaya ringspot virus* (PRSV) and *Watermelon mosaic virus* (WMV) cause huge economic losses (Lecoq & Desbiez, 2012). Out of these notorious Potyviruses ZYMV became a challenge for cucurbits production in Pakistan (Ali *et al*., 2004; Malik *et al*., 2010; Ashfaq *et al*., 2015; Ashfaq & Ahsan, 2017) as well as all over the world since its first record (Lisa *et al*., 1981). It is aphid-transmitted virus and induce vital waves on *Cucurbitaceae* family in Italy, Australia, France, USA, UK, Egypt and in some countries of Africa and Asia (Ashfaq *et al*., 2015; Ashfaq & Ahsan, 2017). ZYMV infected plant produces symptoms like yellowing, mosaic, stunting, shoestrings, and deformation of fruit and seed and pinwheel characteristic to cytoplasmic inclusion bodies in each infected cell (Zechmann *et al*., 2003). The virion of ZYMV is a filamentous particle of 750 nm with a single strand plus sense RNA genome of about 9.6 kb, which encodes a single polyprotein which is furcated by three viral proteinases into ten fully functional proteins likewise to other potyviruses (Adams *et al*., 2005; Lisa *et al*., 1981). These functional proteins include coat protein (CP), cylindrical inclusion protein (CI), the helper component protein (HC-Pro), these aided the virus in movement, combined viral protein genome-linked (VPg)-protease protein, RNA replicases, nuclear inclusion

protein (NIa) serves as a proteinase and b protein (NIb) acts as RNA dependent RNA polymerase (Adams *et al*., 2005; Desbiez & Lecoq, 1997). Serologically detection of viruses is one of the efficient, reliable and economical approach and use frequently for detection (Berniak *et al*., 2009; Mazidah *et al*., 2012; Ashfaq *et al*., 2017a). Traditional nucleic acid based methods such as PCR (Polymerase Chain Reaction) and RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction) could also be used to detect, identify, and classify plant viruses properly, quickly and reliably (Hsu *et al*., 2005; AL-Abedy *et al.,* 2019). Mostly molecular characterization of potyviruses was performed on the basis of nucleotide sequences of coat protein (CP) and cytoplasmic inclusion (CI) gene (Adams *et al*., 2005; Ha *et al*., 2008; Torrance *et al*., 2020; Tsai *et al*., 2008). ZYMV is widely spread in Pakistan and reported by different workers on the basis of nucleotides sequence CP gene (Ali, 2004; Asad *et al*., 2019; Ashfaq & Ahsan, 2017; Ashfaq *et al*., 2015). In this study, ZYMV is characterized in ridge gourd on the basis of the CI gene. Due to the worldwide occurrence of *Potyvirus* epidemics and their potential threat to crop production, rapid and specific protocol for ridge gourd viruses, timely and accurate detection for its management is need of hour. For pursuing this, surveys were conducted in three districts viz; Rawalpindi, Chakwal, and Multan of Punjab to identify and to characterize ZYMV on serological and molecular bases. The information generated by this study would prove fundamental to the development of long-term management strategies against ZYMV at the molecular level.

Materials and Methods

Survey and sample collection: Ridge gourd growing fields of three districts of Punjab viz., Rawalpindi, Chakwal, and Multan were surveyed during growing season 2018-2019 and approximately 300 leaves and fruits samples showing characteristic symptoms of ZYMV such as yellowing, mosaic, shoestring leaves and deformed fruit with the mosaic pattern were collected (Fig. 1). To facilitate return visits, the surveyed sites are located with the Global Positioning System (GPS) coordinates. These collected samples were put into polythene bag on ice bucket and brought to plant virology laboratory and identification of ZYMV positive samples was performed through PTA-ELISA and RT-PCR.

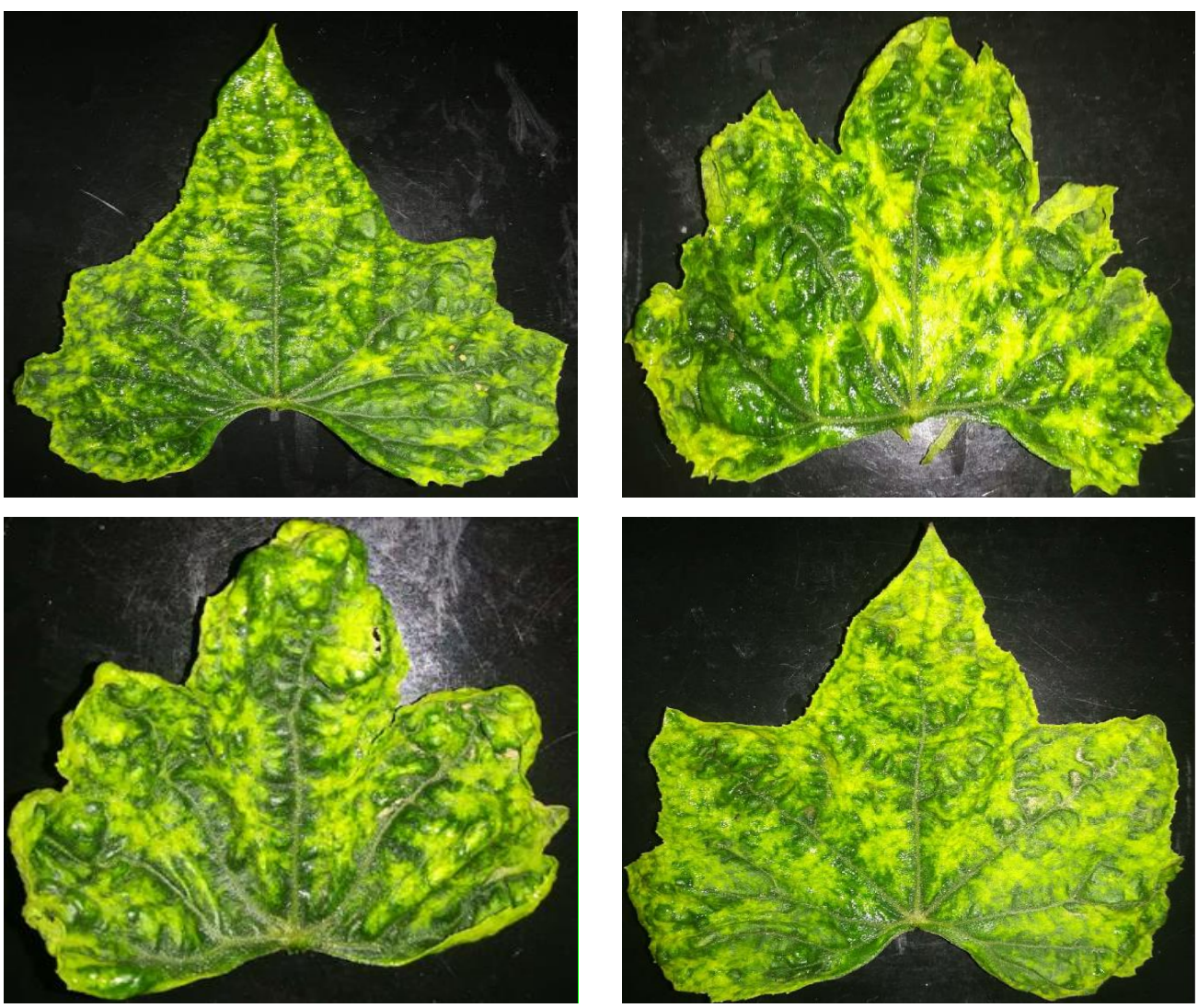

Fig. 1. Symptoms caused by ZYMV in Ridge gourd plants.

Serological assay: To analyze all collected samples on the serological basis for ZYMV both symptomatic leaf and fruit samples along with asymptomatic healthy Ridge gourd leaf samples (control) were exposed to serological diagnosis in duplicate wells with a commercial kit (Cat. No. PSA 27200/0288) following the manufacturer's instructions with some modification of Clark-Adams method (Clark & Adams, 1977). The plant sample was homogenized in extraction buffer and controls were dispensed to each well (200µl per well) and incubated overnight. After washing three times, mouse extracted monoclonal antibody (IgG) diluted 1:1000 in conjugate buffer was appended to the plate wells and incubation of plate was performed at 37°C for 2 h. An enzyme labeled goat anti-mouse antibody alkaline phosphatase conjugate diluted 1:1000 in conjugate buffer was dispensed into well (0.2 mL per well) after three washes and plate was again incubated as described in the previous step.

In the final step, the microtiter plate was coated with the substrate buffer solution (0.2 mL) comprising the dissolved substratum (pNPP tablets). The reaction was analyzed visually for yellow colour development after two hours of incubation and the yellow colour intensity was calculated as optical density (OD_{405nm}) with the help of ELISA reader (HER-480 HT Company (Illford) Ltd, UK). At 405nm, more than the average absorbance value of healthy control samples was considered positive for ZYMV. At each well, the reaction was stopped by adding 50µl of 3 M NaOH. Samples showing mild/light yellow colour were further confirmed by RT-PCR (Reverse transcription-PCR) for authentication either they were infected by ZYMV or not.

Disease incidence: Relative disease incidence was determined based on ELISA results by using the following formula (Rao *et al*., 2002).

$$\%\ \text{D. I. of ZYMV} = \frac{\text{No. of ELISA} + \text{ive samples}}{\text{Total no. of tested Samples}} \text{x} 100$$

Serologically positive sample were used as a source of inoculum for ZYMV in subsequent studies.

Mechanical inoculation: Leaf tissues of ZYMV positive samples were used as a source of inoculum. The ELISA positive samples were mixed at pH 7.2 by using sterilized ice-chilled pestle and mortar in 0.02 M phosphate buffer. Prepared sap was squeezed through cheesecloth. Healthy ridge gourd leaves were dusted with 600 mesh carborundum powder and prepared inoculum/ sap was smoothly inoculated on these healthy leaves with the help of forefinger. After 5 min of inoculation, leaves were washed off with distilled water to wipe out superfluous inoculums. Till the appearance of ZYMV symptoms inoculated ridge gourd plants/ vines and un-inoculated healthy ridge gourd vines were put in an insect free glasshouse under observation.

Reverse transcription polymerase chain reaction (RT-PCR): Total RNA of PTA-ELISA positive samples along with some healthy ridge gourd tissues were extracted by using the TRIzol® Reagent (Cat. No. TR 118 Life Technologies, Carlsbad, USA) enumerated using Nanodrop (Thermo Scientific Co. USA) in line with the manufacturer's instructions. Working RNA dilution at 500 ng/ µL was prepared in nuclease-free water and first-strand complementary DNA (cDNA) was developed by using the Revert Aid RT Reverse Transcription Kit (Cat. No. EP0442 Thermo Fischer Scientific, USA) and CIR 5'-ACICCRTTYTCDATDATRTTIGTIGC-3' (Ha *et al*., 2008) as *Potyvirus* group-specific reverse primer. The subsequent cDNA was used to perform the PCR amplification using the *DreamTaq* Green PCR Master Mix (2X) (Cat. No. K0171 Thermo scientific, USA) and cylindrical inclusion (CI) gene specific forward primers (CIF 5'- GGIVVIGTI GGIWSIGGIAARTCIAC-3' and CIR) (Ha *et al*., 2008) under the following PCR conditions: initial denaturation was performed at 94°C for 3 min; 40 cycles of 94°C for 30sec, 40°C for 45sec and 72°C for 60sec, followed by final extension step of 5 min at 72 °C. PCR products were analyzed by 1.0% (w/v) pre-stained agarose gel by electrophoresis and expected bands were visualized under Gel documentation system. The positive products with ~700bp were purified using GeneJET PCR Purification Kit (Cat. No. K0702 Thermoscientific, USA) and cloned into pTZ57R/T vector (Cat. No. K1213 InsTAcloneTM PCR cloning kit, Fermentas) with chemically competent cells of E. coli strain XL1-Blue. Purification of recombinant plasmid DNA was done by using purification GeneJET Plasmid Kit (Cat. No. K0502, Thermo Fisher Scientific, USA) as described by the manufacturer. Digestion with restriction enzymes (*EcoR*1 and *Hind*III) validate the existence of an insert in transformants and positive clones were sequenced from Macrogen (South Korea) in both orientation using M13 forward and reverse primers. The sequences of ZYMV isolate from *L. acutangula L.* (Ridge gourd) were deposited to GenBank having Accession No. MN897100 and MN897101.

Sequence analysis

Database identity were achieved by means of BLAST (http://www.ncbi.nlm.nih.gov/blast) tool for confirmation of ZYMV. Sequence alignment was performed using Clustal W entrenched in MEGA_X_10.1.7 software (Kumar *et al*., 2018). In BioEdit program version 7.25 (http://www.mbio.ncsu. Edu/ Bio Edit/ bioedit.html) "Sequence identity Matrix" option was used to calculate sequence identities (Hall, 1999). Analyse of 21 nucleotide sequences was performed using the maximum composite likelihood model (Tamura *et al*., 2004). Analyses of 21 amino acid sequences were carried out by the Poisson correction model (Zuckerkandl & Pauling, 1965). Nucleotides and Amino acid-based Phylogenetic trees were developed by the Neighbor-Joining (NJ) method (Saitou & Nei, 1987) using MEGA_X_10.1.7 software with bootstrap analysis of 1000 replicates to characterize the relationships of the sequences. The optimal tree (nucleotide sequences) with the sum of branch length = 0.27352921 and optimal tree (amino acid sequences) with the sum of branch length = 0.08747560, were shown respectively.

Results

Prevalence and incidence of ZYMV: ELISA results revealed that the ZYMV is widely distributed in understudied areas of Punjab; Pakistan not even single site was free from infection. The highest disease incidence of ZYMV (30%) in 2018 was recorded in Rawalpindi followed by Multan (28%) and the least disease incidence was recorded in Chakwal (24%). In 2019, the same pattern of disease incidence was observed. The highest disease incidence was documented in Rawalpindi (32%) followed by Multan (30%) and minimum incidence was recorded in Chakwal 26% (Fig. 2). Out of total 300, ZYMV infected Ridge gourd samples 85 samples showed positive results in PTA-ELISA reaction, with overall 28.33% average disease incidence in understudy districts of Punjab. Out of 100 ZYMV infected samples collected from three different locations of district Rawalpindi 31 samples showed positive reaction for ZYMV. 29 samples out of 100 collected from three different locations of Multan, 25 samples out of 100 collected from three different sites of Chakwal, showed a positive result for ZYMV. Disease Incidence per location during two consecutive years is shown in (Fig. 3).

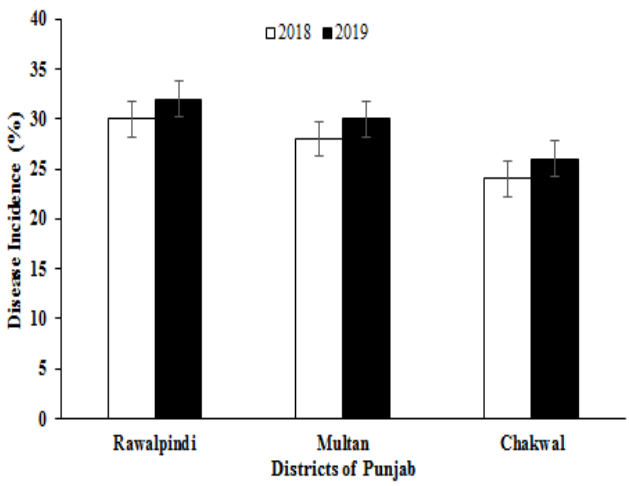

Fig. 2. Relative disease incidence of ZYMV infecting Ridge gourd during 2018-19.

Biannual data of disease incidence showed that in 2019, disease incidence was a little higher compared to 2018. Several factors such as cropping pattern, sowing time, sowing methods, humidity, temperature, aphid population, and time of sample collection are responsible for the aggravation of disease incidence in 2019. Most of the farmers were not familiar with ZYMVD but they were aware of the presence of aphids in their crops (personal communication). Besides, the familiarity regarding aphids, they attributed ZMVD symptoms to water deficiency, aphid feeding damage, and/or crop maturity factor. Many farmers have used insecticides to combat aphid and other insect pests.

Mechanical inoculation: Mechanical inoculation was performed on plants grown in an insect free glasshouse for virus confirmation. Plants showed characteristic symptoms of ZYMV disease (mosaic, yellowing, distortion, and curling) after one week, some plants showed symptoms after two to three weeks of inoculation. Un-inoculated plants showed no disease symptoms. Plants placed in dark pre and post-inoculation expressed more severe symptoms as compared to other plants. This technique was used to identify a virus by its reactions in ridge gourd, and to test the infectivity of virus samples to propagate viruses.

RT-PCR and sequence analysis: Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) amplification of total RNAs acquired from ELISA positive samples was performed by the *Potyvirus* group based degenerate primers CIF/ CIR (Ha *et al*., 2008) specific for CI gene of ZYMV, PCR products each of ~700 bp were recorded from infected tissues. No amplification was seen from healthy controls. After careful analysis, 02 selected partial CI gene sequences of ZYMV isolates (MN897100 and MN897101) from ridge gourd were deposited to GenBank The sequence of both the ZYMV isolate contains 693bp of CI gene. BLASTn tool confirmed that both isolates belong to ZYMV. Sequence analysis revealed that both the new isolates contain *Potyvirus* conserved motif I and V in affirmation with the results of Kadaré & Haenni (1997)) and Ha *et al*., (2008).

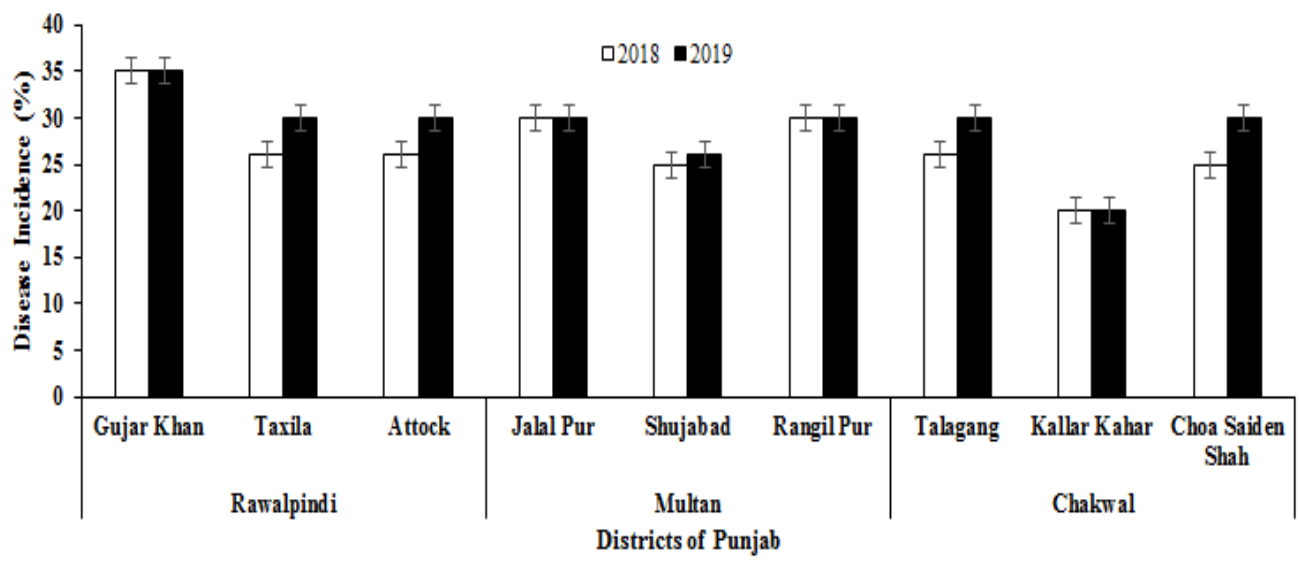

Fig. 3. Disease incidence of ZYMV at different location of three district of Punjab.

Table 1. A list of some reference isolates which are used for the sequence analysis from elsewhere in the world.

Sr. No	Accession No.	Country	Host	Years
1.	KX884570	China	Spiders	2013
2.	MH042026	South Korea	Cucurbita pepo	2016
3.	AY279000	South Korea	Cucurbita moschata	2006
4.	MH042025	South Korea	Cucurbita pepo	2016
5.	KX884565	China	Cray fish	2014
6.	KP828425	Turkey	Cucurbita pepo	2012
7.	AY278999	North Korea	Cucurbita moschata	2006
8.	MH042024	South Korea	Cucurbita pepo	2016
9.	KP828426	Turkey	Cucurbita pepo	2012
10.	MK124612	USA	Pumpkin	2016
11.	MK033874	South Korea	Cucurbita moschata	2006
12.	MH700751	New Guiana	Unknown	2019
13.	AB020477	Japan	Unknown	2016
14.	KX249747	China	Luffa aegyptica	2017
15.	AY278998	South Korea	Cucurbita moschata	2006
16.	KX664482	China	Cucurbita pepo	2016
17.	KX421104	China	Sesamum indicum	2017
18.	AJ515911	China	Water melon	2005
19.	AB369279	South Korea	Melon	2007

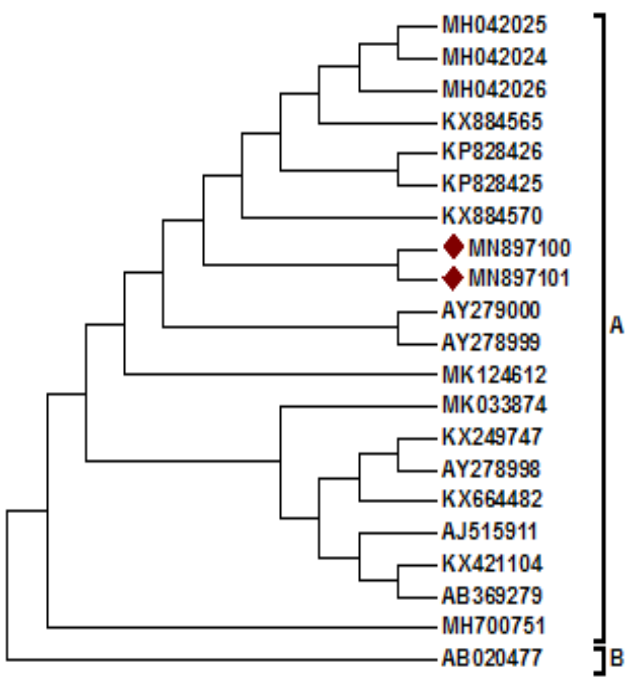

Fig. 4. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.93964058 is shown. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. The analysis involved 21 nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGAX.

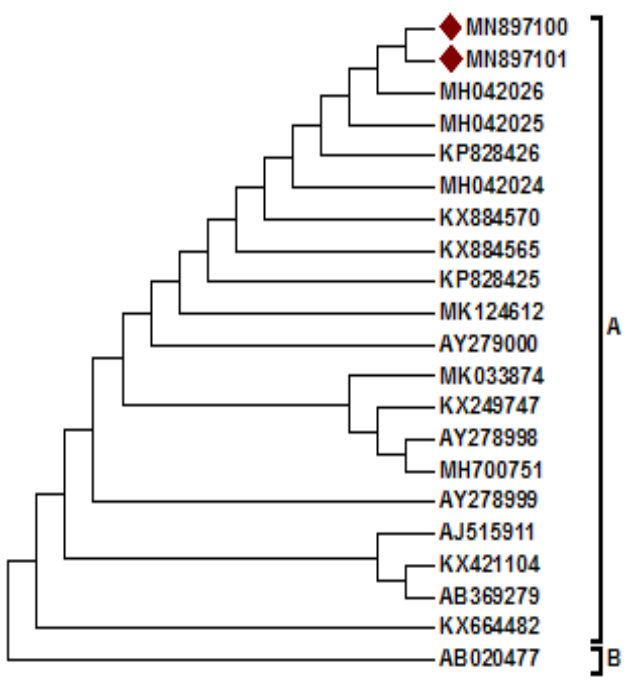

Fig. 5. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 2.99172525 is shown. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 21 amino acid sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGAX.

Phylogenetic analysis and similarity matrix: Sequence identity matrix analysis revealed that both the ZYMV isolates from this study were 98.8% identical to each other. Phylogenetic analysis of current study ZYMV isolates submitted to NCBI was carried out using MEGA X software. The nucleotide sequence of the cytoplasmic inclusion gene of current isolates was aligned with the other 19 ZYMV isolates (Table 1) reported in various geographical regions showed maximum homology after BLAST. The current ZYMV Pakistani isolates (MN897100 and MN897101) show maximum similarity (98%) with MH042026, MH042025 and MH042024 already identified in South Korea (KX884570, and KX884565) from China and KP828426, KP828426 from Turkey. The second highest similarity (97%) was recorded with the isolates MK124612 from USA and AY279000 and AY278999 from South Korea. Out of the 19 ZYMV isolates, KX664482, KX421104, KX249747, and AJ515911 isolated from China MK033874,

AB369279, AY278998 from South Korea showed (<94%) similarity with the Pakistani isolates reported in this study (MH700751, AB020477). Minimum similarity (91.7%) was observed with MH700751and AB020477 isolates from New Guiana and Japan, respectively. Phylogenetic tree analysis based using nucleotide sequences of the CI genes gave two different main clades (A, and B). The Pakistani isolates (MN897100 and MN89701) were present in clad A with other the isolates from China, South Korea, Turkey, New Guiana and the USA. In clad B, isolate from Japan was present (Fig. 4). The CI amino acid sequences were also compared using Mega X sequence alignment tool. The CI amino acid sequence of ZYMV-Pak isolate shared 98%-100% sequence homology with the other isolates identified in the study. Six isolates MH042026, MH042025, MH042024, AY279000, and AY278999 investigated in South Korea KX884565 and KX884570 isolated in China and KP828425 from Turkey shared 100% amino acid sequence homology with the isolates identified in this study. All other isolates shared a 97-98% amino acid sequence identity. Phylogenetic analyses on the basis of amino acid sequences of the CI gene also have two main clades (A, and B). ZYMV-Pakistani isolates (MN897100 and MN89701) were present in clad A with other isolates from China, United state, South Korea, Turkey and New Guiana. Whereas; clad B included the isolates identified in Japan (Fig. 5).

Discussion

Viruses infecting vegetables have always a tremendous threat to sustainable vegetable production throughout the world (Amari *et al*., 2017; Moriones *et al*., 2017). In Pakistan, high incidences of vegetable virus diseases have already been reported by number of scientists (Ashfaq *et al*., 2015; Ashfaq & Ahsan, 2017; Hussain & Atiq, 2017; Tahir *et al*., 2017; Riaz *et al*., 2022). This study demonstrated the pervasive occurrence of ZYMV infecting ridge gourd in three districts viz. Rawalpindi, Chakwal, and Multan of Punjab, Pakistan. ZYMV infecting cucurbits in Pothowar is also previously reported by Ashfaq *et al*., (2015) on the basis of CP gene but in this study, ZYMV infecting ridge gourd on the base of the CI gene was identified. The very first time Southern region of Punjab province was surveyed in this study for the identification of this notorious virus. ZYMV is an important constraint to the successful production of cucurbits in Pakistan as it is reported to infect Bottle gourd (Ali *et al*., 2004), Melon (Malik *et al*., 2010), Round gourd (Ashfaq & Ahsan, 2017) and cucumber (Asad *et al*., 2019). Punjab province is considered as the main hub of agriculture in Pakistan and incidence in this province is alarming for both farmer and economy of the country. Farmer fields were surveyed in three districts of Punjab during 2018 and 2019. Maximum disease incidence (30%) during 2018 was recorded in distrct Rawalpindi, while minimum (24%) was recorded in Chakwal district. During 2019, maximum 32% disease incidence was recorded in the district Rawalpindi whereas minimum (26%) was recored in the district Chakwal .In the district Multan disease incidence was 28% and 30% during 2018 &19, respectively. Samples from 9 different sites of 3 districts were collected and ZYMV infection was detected from every site on the basis of PTA-ELISA results. ELISA results showed that not even a single site was free from infection. Disease incidence varied from region to another region and crop to crop depending upon the climatic conditions of that region and resistance and susceptibility of the crop. In a previous study, ZYMV disease incidence infecting ridge gourd was 40-60% reported by Ashfaq *et al*., (2015). Virus characterization at the molecular level expected to help in proper understanding of genetic composition, recombination, variation, and exact taxonomic status. Some of these techniques are also adopted in this study. ZYMV forms a complex with some other viruses and causes damage to the cucurbits worldwide (Desbiez & Lecoq, 1997).

Most frequently ZYMV produces a synergistic reaction with *Cucumber mosaic virus* (CMV) and as a result of synergy symptoms are more enhanced and severe losses occur than in a single infection of either virus (Fattouh, 2003; Wang *et al*., 2002). The accurate detection by RT-PCR of ZYMV showed the significance of this methodology for the diagnosis of viruses. Molecular characterization by CP and CI gene of ZYMV and other potyviruses are widely used (Wang *et al*., 2006(King *et al*., 2018; Tsai *et al*., 2010; Wang *et al*., 2006). The sequences of the new ZYMV isolates were highly homologous to each other and to the corresponding gene of ZYMV isolates geographically belongs to other countries of the world but they are not 100% identical to each other which clearly indicate that there is a diversity in the isolates of ZYMV in the country. In previous reports, a high level of genetic diversity among ZYMV isolates was observed with in the country (Simmons *et al*., 2008). However, in France it was observed that intra-population indicated a minor change occuring once a ZYMV isolate become established in a vicinity unless a novel virus was introduced. (Lecoq *et al*., 2009) affirming our findings. Moreover, sequence analysis revealed that the presence of *Potyvirus* conserved motif I and V in the new ZYMV-CI isolates was in agreement with the results of Kadaré & Haenni (1997) and Ha *et al*., (2008). Phylogenetic analysis has significant importance in plant viruses study at the molecular level. A phylogenetic relationship based on the CI gene sequences was studied among the new isolates (MN897100 and MN897101), and 19 other ZYMV isolates from elsewhere in the world, as recommended by previous researchers (Chng *et al*., 1997; Ha *et al*., 2008). The CI protein sequence is quite suitable for establishing relationships among *Potyvirus* species since this comparatively large genetic area is the least variable between different species/strains (Oruetxebarria *et al*., 2000). In the present study, nucleotide sequence analysis showed that the Pakistani isolates (MN897100 and MN897101) each shared 98% identity with South Korean and Chinese isolates and present in clad A. Another isolates reported from New Guiana and Japan exhibited less conservative patterns (<92%) with current study Pakistani isolates and showed the maximum distance from the current study ZYMV-Pak isolate included in this study for phylogenetic analysis. These results indicated that origin of current study isolates are Asian. Amino acid-based study of ZYMV-Pak isolate (MN897100, MN897101) showed a conservative pattern

as compared to nucleotide-based analysis. CI gene amino acid sequence of ZYMV-Pak isolate showed 98-100% sequence homology with other ZYMV worldwide reported isolates. ZYMV isolates reported from China, South Korea, and Turkey showed 100% amino acid sequenced based identity with the present isolates. While other isolates reported from the USA, Japan, China, and New Guiana showed less than 99% amino acid sequenced based homology with ZYMV-PAK isolates. A detailed survey of this notorious virus should be carried out to identify the infection in other cucurbits and vegetables. Moreover, the expanding host range of this virus indicates a great threat to crops, which should be tackled using effective viral diagnostic and management approaches.

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