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Full Length Article

Molecular and Biological Characterization of Okra Yellow Vein Mosaic Virus in the Punjab, Pakistan

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Abstract

The study focused on the molecular and biological characterization of OYVMV which helps to understand its transmission efficiency along with genetic diversity and impact on okra varieties. Four okra varieties *viz.,* Sabz Pari, Ujala, Sultan-121, and BS728 were verified for the presence of OYVMV pathogen *via* grafting and insect transmission in biological characterization while PCR was used to detect the presence in molecular characterization. Three- to four-week-old plants were divided into two groups: one inoculated using an insect vector and the other through grafting with PCR-confirmed infected leaf samples. Both groups were compared with the plants used as control for respective transmission studies. The results indicated that grafting was a more efficient transmission method having varied degree of severity for different indicator plants. whereas insect vector transmission showed variability. Virus transmission studies conducted under *in vitro* conditions through whitefly and grafting showed 73.66% and 90% transmission efficiency on "Sultan-121" respectively at 7, 15, and 21 days post-inoculation. Molecular characterization through PCR and gel electrophoresis confirmed the presence of OYVMV with distinct amplified fragments validating viral presence in the infected samples. Sequence analysis of these amplified DNA fragments showed that the OYVMV isolates from these districts share more than 90% genetic homology with previously reported Pakistani strains and have over 85% similarity with isolates from neighboring countries, including India, Bangladesh, Sri Lanka, Malaysia, and China. The study reported that variations in the disease symptoms, incidence and severity were there but not significant in relationship to virus isolates in four geographical locations (Districts). These findings confirm the widespread presence of OYVMV in Pakistan providing critical insights into its transmission mechanisms and genetic structure.

Keywords: OYVMV; Characterization; PCR; PAGE; Phylogenetics; Transmission; Grafting

Introduction

Researchers have developed advanced assays to detect Okra Yellow Vein Mosaic Virus (OYVMV) and confirm its transmission through various modes including insect vectors and vegetative propagation techniques such as grafting and budding (Rathod and Kavya 2023). Vegetative propagation is particularly important as it facilitates the direct transfer of viruses and virus like pathogens from infected to healthy plants (Prabu and Warade 2009). Grafting establishes a direct cambial connection between the rootstock and scion which enhance viral transmission including OYVMV (Ghevariya and Lalit 2017). The primary vector of OYVMV is the whitefly (*Bemisia tabaci*) which transmits the virus in a persistent and circulative manner (Mubeen *et al.* 2021). The virus is ingested by the whitefly and circulates within its body before being transmitted to other healthy plants (Shetty *et al.* 2013). Studies confirmed vector transmission using modern detection methods and emphasized the role of *B. tabaci* as the primary vector (Idrees *et al.* 2024). Mechanical transmission through sap inoculation has not succeeded under standard experimental conditions and seed transmission remains variable with inconsistent findings across different studies (Kumar *et al*. 2017). OYVMV infection cause characteristic symptoms like vein clearing, yellowing and mosaic patterns on leaves leading to severe yield losses (Jamir *et al.* 2020). Mixed infections with other begomoviruses often modify the symptom severity making diagnosis and management even more difficult (Davis and Thompson 2024). Routine detection and characterization of OYVMV rely on molecular techniques especially polymerase chain reaction (PCR) being the most sensitive

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and rapid method available (Patil 2018). PCR-based assays enable the amplification of viral DNA collected from infected plant tissues and allow for precise detection (Mondal *et al.* 2015). PCR is prone to false positives results and need additional confirmations validate results and differentiate true infections from non-specific amplifications (Jiang *et al.* 2024). Polyacrylamide gel electrophoresis (PAGE) is used as a standard follow-up method to validate the results. PAGE not only ensures reliable separation of nucleic acid fragments but also allows for the detailed analysis of viral genomic integrity (Bogiel *et al*. 2022).

There's a lot of research is being done globally on Okra Yellow Vein Mosaic Virus (OYVMV) which especially focus on how it spreads and how we can manage it. In Pakistan okra is an important crop but there has been little research about the virus, especially when it comes to understanding how it behaves differently in different areas or how it varies genetically. This gap is significant because OYVMV is a major threat to okra production in Pakistan and is an important part of the economy. This study aimed to change that by taking a closer look at how OYVMV behaves in different fields of Pakistan in different areas. By analyzing the genetic makeup of virus and how it spreads, we have a better understanding of its diversity and how it changes over time in specific environments. This knowledge is a key to developing better and more effective ways to manage the disease including breeding okra varieties that are more resistant to the virus. We can make future monitoring faster and more reliable by improving the detection and tracking of the virus using techniques like PCR and gel electrophoresis. Ultimately, this research will help protect okra production and provide valuable insights that can guide future studies and control measures.

Materials and Methods

Detection of OYVMV

Samples of okra leaves from the Sargodha Division were collected and subjected to the diagnosis of OYVMV *via* nucleic acid base techniques: PCR, among others. Already published primers (Senevirathna *et al.* 2016) were used in this study with the expected size of 800 bp:

F 5'- ATG TCG AAG CGA GCT GCC G 3'
R 5'-YCA ATT CGT TAC AGA GTC ATA AA 3'

Nucleic acid extraction

DNA was extracted from plant tissue by grinding it in a mortar and pestle with liquid nitrogen to create a fine powder. The tissue was then mixed with a 2% CTAB extraction buffer (500 μL for 100 mg tissue) and vortexed. The mixture was incubated in a water bath at 60°C for 30 min and then centrifuged at 14,000 xg for 5 min. The supernatant was transferred to a new tube and treated with RNase A for 20 min at 37°C and then mixed with an equal volume of chloroform/isoamyl alcohol (24:1). After centrifugation to separate the phases, the upper liquid phase was collected and this step was repeated until the solution was clear. DNA was precipitated by adding chilled isopropanol (0.7 volumes), then incubated at -20°C for 15 min and then centrifuged. The pellet was washed with ice-cold 70% ethanol, dried, and resuspended in 20 μL TE buffer (10 m*M* Tris, pH 8.0 and 1 m*M* EDTA) (Doyle and Doyle 1987; Gupta *et al.* 2020).

PCR for genome amplification

PCR was successfully employed to detect the virus genome and its variants in infected plants. These methods allow the simultaneous detection of multiple strains of the virus, improving diagnostic efficiency (Priyanka *et al.* 2024).
Conditions for PCR will be followed:
Initial denaturation at 94°C for 3 min (1 cycle) at step 1.
Denaturation at 94°C for 1 min (35 cycles).
Annealing at 55°C for 45 sec and extension at 72°C for 90 sec.
Final extension at 72 °C for 15 min (1 cycle).
The product was analyzed by gel electrophoresis for PCR products.

Agarose gel electrophoresis

In order to perform gel electrophoresis runs according to the procedure mentioned by Senevirathna *et al.* (2016) and Kumari *et al.* (2021). A 1.5% agarose gel solution was prepared in 0.5X TBE buffer to perform gel electrophoresis and heated until the agarose dissolved. After cooling, ethidium bromide (0.5 μg/mL) was added to stain the DNA. The gel solution was poured into a casting tray with a comb to form wells and allowed to solidify. PCR products (5 μL) were mixed with 1 μL of 6X loading dye and loaded into the wells. The gel was placed in the electrophoresis chamber while covered with 0.5 X TBE buffer and electrophoresed at 70V for one h. The gel was removed and visualized using a UV trans-illuminator and image of the gel was taken using a gel documentation system to analyze the DNA fragments.

Phylogenetic analysis

The positive clones identified as Okra Yellow Vein Mosaic Virus (OYVMV) were submitted for sequencing. Resulting sequences were analyzed using the BLASTn tool at NCBI to confirm viral identity. Multiple sequence alignment and phylogenetic analysis were conducted using MEGA 6.1. The phylogenetic tree was constructed using the maximum likelihood (ML) method and bootstrap analysis with multiple replications performed to assess the reliability of the tree topology.

Graft and insect transmission

The transmission studies in this research were carried out on the OYVMV among four okra varieties namely Sabz Pari, Ujala, Sultan-121 and BS728. The transmission was studied

through the graft and insect vector inoculation methods. For each variety, 15 inoculated and 5 control plants were taken from a total of 20 indicator plants. Ten of the fifteen inoculated plants have been subjected to graft inoculation (Fig. 1), while five process exposed to an insect vector. Infected sources are PCR-confirmed okra leaf samples, yielding 800-bp amplicons as described by Senevirathna *et al.* (2016) and Jitu *et al.* (2021). Two assays of transmission were done in 3 to 4-week-old okra plants, showing that graft inoculation was more efficient than insect vector inoculation. Sultan-121 and Ujala plants were found more susceptible to OYVMV with symptoms such as mild mosaic vein clearing, dwarfing, and curling of leaves (Ahmed and Ladan 2022) after 7, 15, and 21 days after inoculation (Malathi *et al.* 2017). Insect vector trials in which viruliferous insects were collected using aspirators then kept in cages constructed of wooden material with galvanization insect proof mesh (Mohanan *et al.* 2020). This environment was maintained at 30°C with 60% relative humidity. Insects had a 24 h acquiescence period to gain the virus from infected plants before a 24 h inoculation access period for allowing healthy plants to acquire the virus. The inoculated plants were kept in an insect-proof glasshouse until symptom development, as per (Venkataravanappa *et al.* 2012, 2013). To confirm OYVMV transmission and graft healthy plants infected with it (Naveed *et al.* 2023), effective grafting technique inoculation was employed . Ten plants of each okra variety were borne on infected grafts with five controls. Infected tested samples were confirmed by PCR and all plants grown in insect-free greenhouse conditions of 28-32°C for symptom observation. These results correlated with the experiments in vegetative propagation by Ghevariya and Lalit (2017), in that five commercial okra varieties were tested under controlled conditions (Fig. 5-6).

Fig. 1: Different steps of Graft inoculation of OYVMV on okra plants

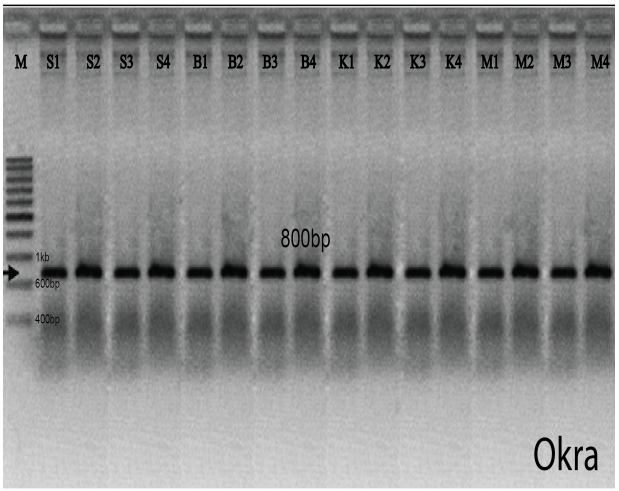

Fig. 2: PCR detected the OYVMV in different Okra samples (Lane M: Marker; S 1-4: OYVMV in Sargodha samples; B 1-4: OYVMV in Bhakkar samples; K 1-4: OYVMV in Khushab samples; M 1-4: OYVMV in Mianwali samples; S1-Chak 128 NB, S2 = Chak 104 SB, S3 = Chak 136 NB, S4 = Sial Sharif, B1 = Kotla Jam, B2 = Deullewala, B3 = Jhok Mehar Shah, B4 = Gohar Wala, K1 = Padhrar, K2 = Jauharabad, K3 = Mitha Tiwana, K4 = Naushehra, M1 = Musakhel, M2 = Rokhri, M3 = Harnoli, M4 = Chashma)

Results

Molecular characterization of OYVMV

The PCR analysis of okra leaf samples collected from various localities in the Sargodha Division produced distinct ~800 bp amplicons in molecular detection and confirmed the presence of OYVMV in the infected samples. These PCR results were confirmed by polyacrylamide agarose gel electrophoresis, which consistently showed clear and specific bands corresponding to the virus (Fig. 2). Sequence analysis of these amplified DNA fragments showed that the OYVMV isolates from these districts share more than 90% genetic homology with previously reported Pakistani strains and have over 85% similarity with isolates from neighboring countries, including India, Bangladesh, Sri Lanka, Malaysia, and China. Phylogenetic analysis further exhibited that these Asian isolates cluster closely together which indicate the high degree of genetic conservation among them (Fig. 3).

Biological characterization of OYVMV

In transmission studies conducted on indicator plants of four okra cultivators (Sabz Pari, Ujala, Sultan-121 and BS728), both graft inoculation and insect (whitefly) vector methods were used. In the whitefly-mediated transmission trials, the infection percentages varied among the cultivars, with Sultan-121 showed an infection rate of 73.33%, followed by Ujala at 60%, Sabz Pari at 53.33% and BS728 at 46.66% (Table 1). In contrast, graft inoculation exhibited higher transmission rates, with Sultan-121 reaching 90%, Ujala 80%, BS728 60%, and Sabz Pari 50% infection (Table 2).

Table 1: Insect vector transmission of OYVMV on different okra varieties excluding control

Sr. No.	Variety	No. of Test group plants	No. of plants with successful Transmission followed by PCR confirmation	No. of plants with no symptoms or died	Infection%
1	Sultan-121	20 (15 + 5 control)	11	3 died + 6 with no symptoms including control	73.33
2	Ujala	20 (15 + 5 control)	9	4 died + 7 with no symptoms including control	60
3	Sabz Pari	20 (15 + 5 control)	8	3 died + 9 with no symptoms including control	53.33
4	BS 728	20 (15 + 5 control)	7	4 died + 9 plants showed no symptoms including control	46.66

Table 2: Graft transmission of OYVMV on different okra varieties excluding control

Sr. No.	Variety	No. of Test group plants	No. of plants with successful grafted followed by PCR confirmation	No. of plants with no symptoms or died	Infection%
1	Sultan-121	15 (10 + 5 control)	9	2 died + 4 with no symptoms including control	90
2	Ujala	15 (10 + 5 control)	8	3 died + 4 with no symptoms including control	80
3	BS728	15 (10 + 5 control)	6	3 died + 6 with no symptoms including control	60
4	Sabz Pari	15 (10 + 5 control)	5	5 died + 5 plants showed no symptoms including control	50

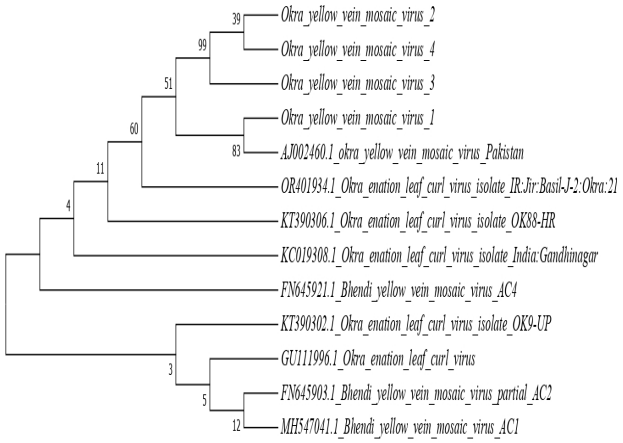

Fig. 3: Phylogenetic analysis of OYVMV Isolates from Sargodha (1), Bhakkar (2), Khushab (3) and Mianwali (4) using maximum likelihood method

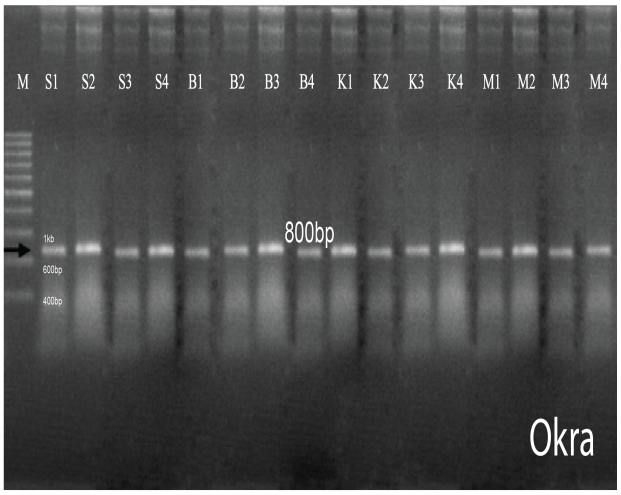

Fig. 4: Bands of detected OYVMV in different Okra samples (Lane M: Marker; S 1-4: OYVMV in Sargodha samples; B 1-4: OYVMV in Bhakkar samples; K 1-4: OYVMV in Khushab samples; M 1-4: OYVMV in Mianwali samples; S1-Chak 128 NB, S2 = Chak 104 SB, S3 = Chak 136 NB, S4 = Sial Sharif, B1 = Kotla Jam, B2 = Deullewala, B3 = Jhok Mehar Shah, B4 = Gohar Wala, K1 = Padhrar, K2 = Jauharabad, K3 = Mitha Tiwana, K4 = Naushehra, M1 = Musakhel, M2 = Rokhri, M3 = Harnoli, M4 = Chashma)

Symptoms such as vein clearing, yellowing, mosaic patterns, and leaf curling were observed at 7, 15 and 21 days post-inoculation and all symptomatic plants were subsequently confirmed by PCR analysis for viral presence (Fig. 4).

Discussion

This study provides a comprehensive knowledge about the transmission, molecular detection, and genetic diversity of OYVMV in different okra cultivars. Our results showed that graft inoculation achieves a significantly higher transmission rate up to 90% in Sultan-121 compared to insect vector (whitefly) inoculation with maximum rates around 73.33%. These findings are consistent with earlier studies that have reported grafting as a highly efficient method for viral transmission due to the direct vascular and cambium connection between infected and healthy tissues (Ghevariya and Lalit 2017). Grafting has been recognized as a method that facilitates virus movement through vascular tissue and bypass some of the inconsistencies observed with insect-mediated transmission (Chang and Chen 2024). The variability observed in whitefly mediated transmission, which aligns with the inherent inconsistencies reported by Shetty *et al.* (2013) and Kumar *et al.* (2017), where different factors such as vector behavior, feeding time and environmental conditions play important roles in transmission efficiency. This comparative performance results supports the concept that while both methods are viable, grafting may offer more predictable outcomes for controlled inoculation. The observed differences among the tested cultivars like Sultan-121 and Ujala were more susceptible to OYVMV compared to BS728 and Sabz Pari are in line with previous literature. Appiah *et al.* (2020) and Idrees *et al.* (2024) have reported similar cultivar dependent variations in symptoms and infection rates. These studies suggest that genetic makeup of the okra cultivars influence susceptibility and this trend is clearly evident in our data.

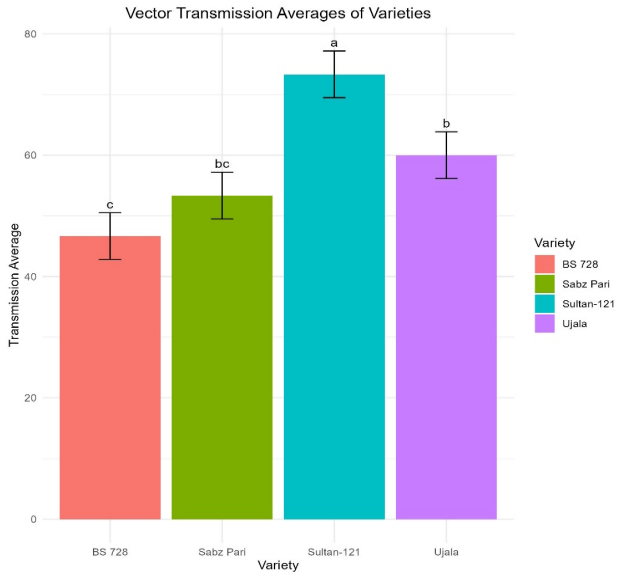

Fig. 5: Insect vector transmission of OYVMV on different okra varieties

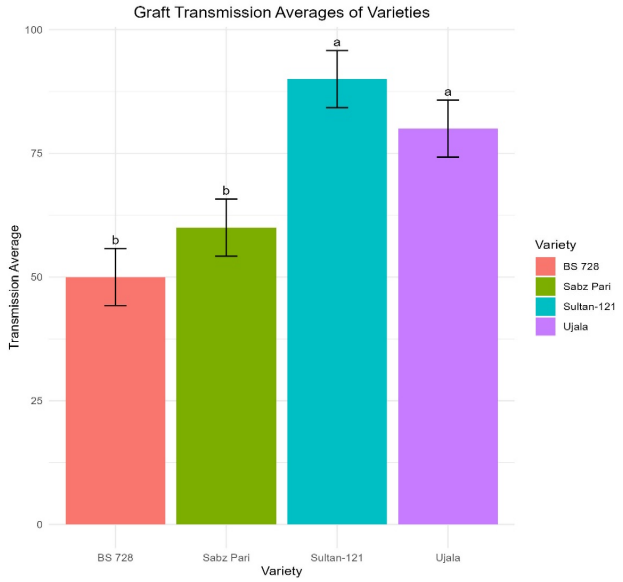

Fig. 6: Graft transmission of OYVMV on different okra varieties

The presence of characteristic in the more susceptible cultivars confirms earlier observations where host genetics play significant role in disease development (Singh *et al.* 2016; Mubeen *et al.* 2017). Furthermore, variation in susceptibility among cultivars is not only a result of genetic factors but also environmental influences such as soil composition and climate conditions which can either enhance or mitigate the symptoms and progression of the disease (Jevtić *et al.* 2020). Molecular characterization using PCR and gel electrophoresis gave clear evidence of OYVMV presence by showing the ~800 bp amplicons. These results are in in accordance with the work of (Senevirathna *et al.* 2016) who successfully employed similar primer sets for the specific detection of OYVMV. Sequence analysis and phylogenetic evaluation revealed high genetic similarity which is more than 90% homology among Pakistani isolates and over 85% similarity with isolates from neighboring countries. These results are aligned with earlier reports which suggest that the OYVMV population in the region is relatively stable (Prabu and Warade 2009; Mubeen *et al.* 2017). The low genetic variation found in different areas is may be due to either a recent common origin or ongoing gene flow due to regional agricultural practices (Kumar *et al.* 2017; Tharmila *et al.* 2017). The stable genetic structure of OYVMV in the region has important implications for disease management strategies and indicated that regional practices may be broadly applicable for controlling viral spread (Zhang *et al.* 2020). The comparative efficiency of grafting offers a reliable method for virus transmission in controlled experiments which can be used diagnostic and detection in host-virus interactions (Wasala *et al.* 2019). Moreover, the ability to achieve consistent transmission through grafting makes it an ideal model for studying host-virus interactions in a controlled environment (Chang and Chen 2024). Given the high homology among OYVMV isolates, the management approaches developed for one region may be applicable across other areas. These findings align with the studies which emphasize on precise detection methods and understanding transmission dynamics to mitigate the OYVMV disease (Ravisankar 2002; Bilqees *et al.* 2020). The continued development of molecular tools and techniques will further enhance the ability to manage this disease and reduce its impact on crop yields.

Conclusion

This study provides valuable insights into the molecular and biological characteristics of Okra Yellow Vein Mosaic Virus (OYVMV) in Pakistan. Grafting was found to be a more efficient transmission method than whitefly vector-mediated transmission with Sultan-121 showing the highest transmission rates. Molecular characterization using PCR confirmed the presence of OYVMV in various okra varieties and sequence analysis revealed high genetic similarity among local isolates and those from neighboring countries. The study also highlighted cultivar-specific susceptibility with varieties like Sultan-121 and Ujala being more susceptible to OYVMV. Overall, the research confirms the widespread presence of OYVMV in Pakistan, reveals its transmission mechanisms and offers insights into its genetic structure. These findings are crucial for developing effective disease management strategies including breeding resistant cultivars and improving early detection methods for better control of the virus.

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Author Contributions

MAS: Conducted the research trial and writing-original draft. YI: Conceptualized, validation, and supervised the trial. MAZ: Co-supervised the trial. MM: Statistical analysis, Finalization and writing-original draft. TS, KA and AS: Helped technical assistance for lab analysis.

Conflict of Interest

All authors declare no conflict of interest.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable to this paper.

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