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# Expression of Key Genes Related to Yellow Vein Mosaic Virus Resistance in Okra Via qRT-PCR

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***Authors' contributions***

*This work was carried out in collaboration among all authors. Author MK managed the literature searches, and wrote the first draft of the manuscript, performed the statistical analysis, author NKS designed the experiment and performed manuscript editing, author MP provided the raw material. Author AS assisted in virus inoculations experiment. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aims:** The present study was based on quantitative real time gene expression analysis on the elite cultivar *Abelmoschus esculentus* Punjab Padmini (susceptible) and wild species *Abelmoschus moschatus* accession 140986 (resistant) in response to yellow vein mosaic virus disease in okra.
**Study Design:** The staggered sowing of both species was done to coincide the stage of virus inoculation at two true leaf stage.

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**Place and Duration of Study:** School of Agricultural Biotechnology and Department of Vegetable Sciences, Punjab Agricultural University, Ludhiana.
**Methodology:** The virus inoculations with viruliferous whiteflies was done, followed by leaf sampling at 0, 1, 3, 5, 10, 15, 20, 25 days post inoculation to know the virus titer at different time points.
**Results:** Though the disease symptoms appeared near leaf margins at 5 days post inoculations yet quantitative real time PCR method was used to measure the relative gene expression of begomovirus coat protein gene. The observations suggest that the virus titer tends to increase at 5 days post inoculations to the higher extent in both species. Interestingly, the maximum fold of begomovirus specific gene expression at 5dpi was observed in resistant species. At five days inoculation time period in the resistant species, the virus replication tends to enhance to resist the viral attack against the strong immune response of resistant species.
**Conclusion:** Our observations confound with the flor-flor hypothesis therefore; we suggest that 5 dpi is recommended for screening of YVMV disease at the gene and genomic levels.

*Keywords: Yellow vein mosaic virus; Abelmoschus esculentus; quantitative real-time PCR; whitefly; gene expression.*

## 1. INTRODUCTION

Okra (*Abelmoschus esculentus* (L.) Moench) belonging to Malvaceae family is a traditional annual vegetable crop grown in tropical, subtropical and Mediterranean climatic zones (Rao et al., 2019). The economically important crop is widely known for its ease of cultivation and adaptability to varying moisture conditions (Kumar et al., 2017). Worldwide, the total okra cultivation area is 0.35 million hectares, with a production of 3.5 million tons and a productivity of 9.6 million per hectare (https://www.agrigoaexpert.res.in/). The growth and productivity are severely affected by various destructive pathogens like bacteria, fungi and virus. The major objective of okra breeding is to increase the crop yield with minimal loss due to insect pest infections (Chowdhury & Kumar 2019). Okra is a significant vegetable crop consumed throughout India, and YVMV is the most destructive disease that causes severe losses. Thus, it is necessary to gather in-depth knowledge about host-pathogen interactions in order to develop management strategies and breeding programmes.

Among the viral diseases, yellow vein mosaic virus (YVMV) is the most destructive viral disease of okra caused by monopartite, bipartite begomovirus and associated satellites, with a reported yield loss of 80-94 percent under heavy infection (Kumar et al., 2017). So, management of this viral disease is a prerequisite for the yield improvement. First reported in 1924 (Kulkarni, 1924), YVMV is believed to have originated in India (Shetty et al., 2013). The YVMV disease is neither sap transmissible nor seed transmissible. The only mode of YVMV virus transmission is through the insect vector, whitefly (*Bemisia tabaci* Genn.) in nature (Kumar et al., 2017). This disease has become a limiting factor in the successful cultivation of this crop because loss in yield, due to YVMV in okra was found ranging from 30 to 100% depending on the age of the plant at the time of infection (Singh 1996). It is characterized by different degrees of chlorosis and yellowing of veins and veinlets, reduced leaf size, number of fruits, and stunting (Venkataravanappa et al., 2012). The fruit reduction of up to 96% has been reported with the incidence of YVMV disease at the early stage (Shetty et al., 2013).

Till date, no work has been reported for gene expression study of yellow vein mosaic virus disease in Indian germplasm of okra. Quantitative real time PCR serves as a promising approach to measure the mRNA levels with high degree of accuracy and sensitivity (Hürkan et al., 2018). The normalization of qRT-PCR is a critical step with the aim to reduce the sampling errors and equalization of the template amount (Radonic et al., 2004). The reference (tubulin) and target (begomovirus specific coat protein) genes have been employed to analyze the virus titer in data time points that delineated an accurate and normalized relative gene expression in response to YVMV disease. The normalization of genes of interest and reference gene expression data determined the variations in input in different biological samples in three replications (Abdallah & Baurer 2016). The inclusion of reference gene employed the similar expression in all cells and tissue types on different plant development stages and different

time data points (Resetic et al., 2013). Quantitative Real time PCR (qRT-PCR) eases the detection of numerous plant viruses with RNA or DNA genomes (Ammara et al., 2017; Bester et al., 2014; Rao and Sun 2015; Shafiq et al., 2017; Tharmila et al., 2019). Unlike fungicides and bactericides, no commercial viricides have yet been developed; therefore, viral diseases are not amenable to control by any direct methods (Thresh 2006).

The host resistance is the most reliable mechanism to manage this devastating disease because chemical method employed for controlling this disease gain very limited success due to their non-permanent nature. The wild *Abelmoschus* species are highly valuable sources of disease resistance genes (Singh et al., 2007) so, the wild species screening against virus resistance considered as a regular practice in breeding programme at Department of Vegetable Science, Punjab Agricultural University. Since the last couple of years, we at Punjab Agricultural University screened more than 400 elite okra lines and identified YVMV resistant accessions of different *Abelmoschus* species namely *A. moschatus*, *A. angulosus* and *A. manihot* subspecies manihot (Pathak and Bal 2008). The method of improving disease resistance includes screening of source of resistance in wild as well cultivated species (Shetty et al., 2013). The genetics of YVMV resistance reported to control by two dominant genes, single dominant gene, complementary and duplicate gene effects (Shetty et al., 2013) that complicate the understanding of the virus resistance mechanism. Moreover, source of virus resistance is limited in the cultivated species of okra (Kumar et al., 2017).

The present study comprised of *A. esculentus* cultivar Punjab Padmini (Susceptible) and *A. moschatus* accession 140986 (Resistant) and exploited the use of SYBR Green-based fluorescence qRT-PCR assay which is measured after each cycle (real-time). The expression analysis has the potential to identify the physiological context in which a gene is transcribed and the encoded protein produced in the cell (Abdallah & Baurer 2016). The increasing levels of fluorescence during the qRT-PCR showed the double-stranded PCR fragments amplification. The selection of reference gene for qRT-PCR expression analysis provide fundamental base for elucidating the molecular mechanisms (Rasheed and Beevey 2024, Soorni et al 2024). The “quantification cycle” value of a qRT-PCR run is used for quantification that reflects the time point and cycle during the exponential PCR phase at a specific threshold level of SYBR Green fluorescence. This is the first report pertaining to analyse the gene expression study of YVMV disease in okra.

## 2. MATERIALS AND METHODS

### 2.1 Plant Material

Screening against virus resistance is a regular practice in breeding programme at Department of Vegetable Science, Punjab Agricultural University. *Abelmoschus* germplasm has been screened against YVMV resistance and none of the lines of cultivated species was resistant but among *Abelmoschus* species namely *A. moschatus*, *A. angulosus* and *A. manihot* subspecies manihot were identified to have high level of YVMV resistance (Pathak & Bal 2008). The elite cultivar Punjab Padmini which although is under the farmers cultivation area but it is prone to YVMV disease. The experiment was conducted on these both resistant and susceptible okra species in the experimental field areas of School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana. Punjab Padmini exhibit sparse stem pubescence, medium depth of leaf lobbing with purple petal color on both sides and green stem. On the other hand, resistant species exhibit high stem pubescence, shallow depth of leaf lobbing with purple petal base color inside only and reddish green stem.

### 2.2 Sowing of Plant Material

The seeds of both species were obtained from the Department of Vegetable science, Punjab Agricultural University. It has been observed that wild okra species germinated 10-15 days later as compared to the cultivated species. Therefore, the staggered sowing as well as pre-treatment with 0.1% sucrose was done in both okra species. This was done to coincide the stages for virus inoculations at two true leaf stage in both the species. Both species were raised using standard agronomical practices.

### 2.3 Virus Inoculations

The virus inoculations at two true leaf stage were given to all plants using whitefly as a vector. The non-viruliferous whiteflies (*Bemisia tabaci*) were reared as a control in an insect proof cage on the cotton (*Gossypium hirsutum L.*) species

maintained at 25–30°C temperature and 50 per cent humidity. These virus-free whiteflies were collected by gentle shaking from the lower surface of leaf. The whiteflies were initially allowed to feed on the YVMV virus infected susceptible plant “Punjab Padmini” as a primary source of virus for virus acquisition access of 24 hours period followed by virus inoculation access period on all plants (Fig. 1).

## 2.4 RNA Extraction and cDNA Synthesis

RNA was extracted from each leaf sample of both species at 0, 1, 3, 5, 10, 15, 20 and 25 dpi in three replications of control and treated plants using RNA Isolation kit according to manufacturer instructions (Qiagen). Eight time points were selected based on crop stages. Initial time point of 0 day represented the control while other days symbolised the disease development assessment. Total RNA quality and quantity were determined in 1.5 % agarose gel electrophoresis. First strand cDNA was synthesized using High-capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer’s instructions. Then, the 20 µL reaction subjected to thermal cycler for reverse transcription with 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes and hold at 4°C.

## 2.5 PCR Amplification using Reference and Target Primers

The extracted cDNA undergoes normalization at 50ng/µL for further analysis, which was confirmed through PCR amplification using reference tubulin primer which is an internal constitutively expressed control primer and begomovirus specific coat protein (target) gene specific primers. The amplicon size of tubulin and begomovirus specific coat protein primer was 100 bp and 137 bp respectively. Tubulin is the most widely used reference gene to analyse the gene expression pattern in okra (Zhang et al., 2022; Zhu et al., 2023). The sequences of primers are shown in Table 1. The relative gene expression of virus using both target and reference primers were analysed through real time PCR reaction.

## 2.6 Quantitative Real-Time PCR

Each real-time PCR was performed in three replicates with a 10- µL final volume on an applied biosystems ABI real-time PCR. The qRT-PCR analysis was conducted on biological as well technical replicates of both the species in three replications of control and treated samples. The RT-qPCR includes steps as follows: initial denaturation at 95°C followed by 40 cycles at 95°C for 1 minute, 62°C for 1 minute and 72°C for 1 minute. The standard dilutions, controls without templates and inoculated samples were run in triplicates to measure the fluorescence signals at the end of each extension step. The threshold cycle (Ct) for each sample was calculated using the exponential growth phase and the baseline signal from the fluorescence versus cycle number plots. The melt curve analysis was performed on the PCR products at the end of each PCR run to access the amplification of single product. The normalized ΔCt data of viral gene specific primer and tubulin specific primers were used to calculate the relative gene expression fold change (Livak & Schmittgen 2001; Schmittgen & Livak 2008).

The relative expression of viral gene in resistant and susceptible plants indicated the variation of infection in these two plants types and signifies the virus proliferation. The deviations for both the target and internal control genes determined to calculate the fold change in the target gene for each sample using the equations $2^{-\Delta\Delta CT}$ method (Schmittgen & Livak 2008). The statistical analysis was performed to measure the fold change and standard deviation of three replication data using SAS software (Yuan et al., 2006).

**Table 1. Primer sequence of begomovirus specific (target) and tubulin (reference) genes***

| Primer* | Forward primer | Reverse primer |
|---|---|---|
| Begomovirus coat protein gene | 5' GCCYATRTAYAGRAAGCCMAG 3' | 5' GGRTTDGARGCATGHGTACATG 3' |
| Tubulin gene | 5' AGGAGGATGCAGCCAACAAC 3 | 5' CTGATACGGTCCAGGCATAGG 3' |

**Begomovirus specific primers are degenerate primers, H is for A/C/T, R for G/A, Y for C/T, M for A/C, D for G/A/T and N for A/T/G/C*

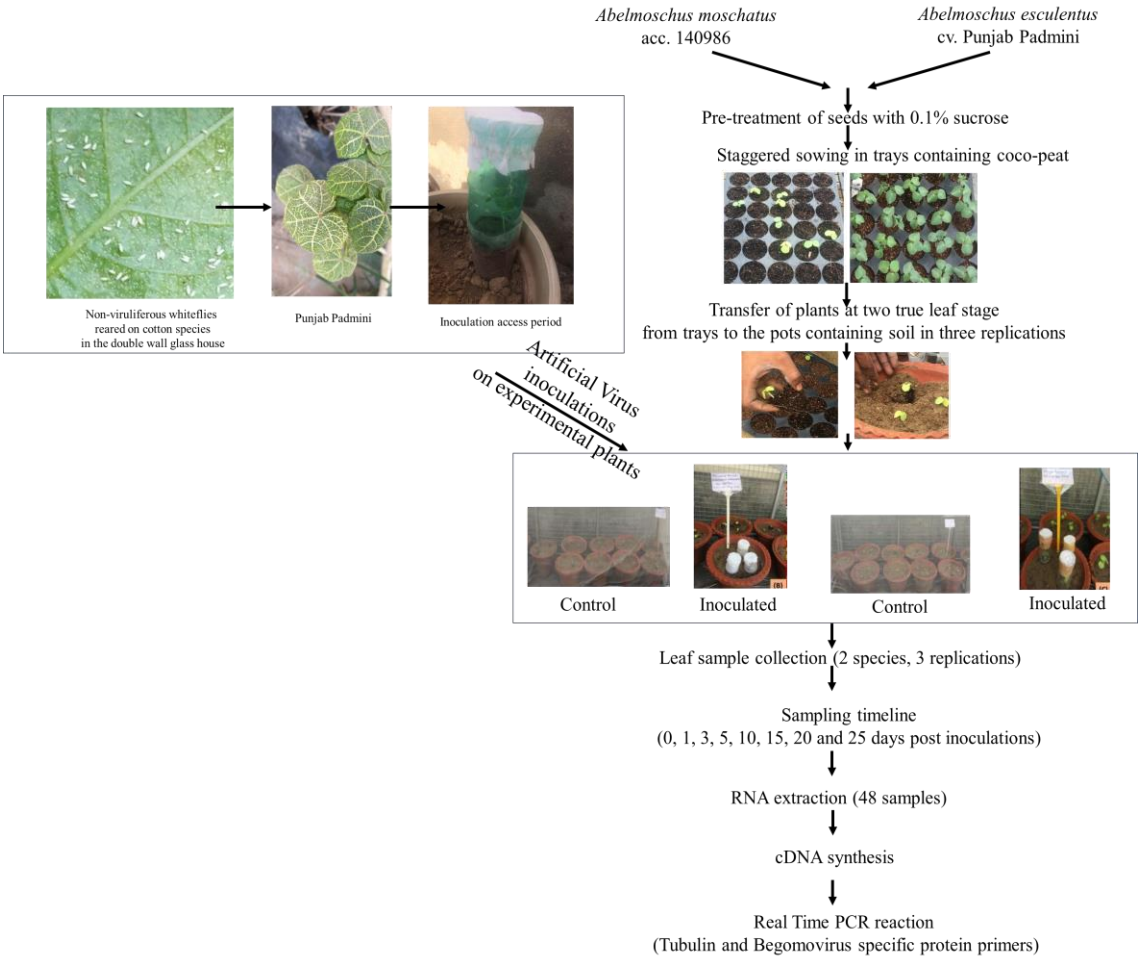


**Fig. 1. Schematic representation of the experimental layout, including sowing, inoculation, and sampling timelines**

## 3. RESULTS AND DISCUSSION

### 3.1 YVMV Disease Symptoms

In the present study, the begomovirus titer as well as the disease symptoms were observed at different time points of virus inoculation. For virus inoculations, staggered sowing was done to coincide the stage of virus inoculations at two true leaf stage in both species as the wild species took 10-50 days more for germination than the cultivated species. The resistant species showed less germination with weak growth as compared to the cultivated genotype (Susceptible) with green leaves and healthy seedlings. It is well known that resistance in okra is not due to the escape (Shetty et al., 2013) and the translocation of virus is intrinsic to whitefly (Czosnek and Brown 2009) that showed that YVMY depends on the presence of whitefly in the surroundings (Kumar et al., 2017).

Fifty seeds of both the species were sown and it has been observed that cultivated species showed 100% germination while wild species showed less germination (5%) as compared to cultivated species. The disease symptoms were recorded on the basis of the visible appearance of yellow veins on the leaf surface up to plant maturity. YVMV disease symptoms appeared at two true leaf stage rather than cotyledonary stage inoculations.

The stunted growth of the plant in the susceptible okra cultivar was also observed. YVMV symptoms started appearing at 5-7 dpi in susceptible while resistant species showed no disease symptoms even at 10 dpi (Fig. 2 A, B). YVMV disease screening showed that susceptible species showed visible yellow vein clearing symptoms on the leaves at 30 dpi while resistant species showed no YVMV symptoms even upto 30 dpi (Fig. 2 C, D). The last time

points showed the maximum disease development. The present study confirms that the resistant species remain as stay green phenotype up to maturity.

The appearance of interveinal clearing in response to YVMV disease starts from five days post inoculations near the leaf margins and visible appearance of vein yellowing occurs at ten days post inoculations in the susceptible species. The susceptible plants within 30 dpi showed severe infection with YVMV disease (Fig. 2 C) due to systematic viral movement in the plant system. The viral diseases viz. alfalfa mosaic virus, cucumber mosaic virus, tomato spotted wilt virus and tobacco mosaic virus also showed high peak virus concentration at 10 days after infecting in the different plant hosts (Xie and Lin 2004; Heet al., 2013). Senevirathna et al. 2016 also observed that chlorosis may extend to the inter-veinal area and may result in complete yellowing of leaves.

## 3.2 Relative Gene Expression Analysis through Qrt-PCR

To know the virus titer at different time points of dpi of virus in both the species, the gene expression analysis was conducted. Although interveinal chlorosis symptoms appeared after 10 dpi in the susceptible species and no symptoms were observed in the resistant species upto 30 days. To analyse the virus titer in response to days to inoculation, we performed cDNA amplification using tubulin and begomovirus specific coat protein gene specific primers and run on agarose gel electrophoresis system (Fig 3). After confirmation of 100 bp and 137 bp amplicon size of tubulin and begomovirus specific coat protein, then we opted qRT-PCR to analyse the virus titer of begomovirus specific coat protein gene expression study in response to YVMV disease in okra. qRT-PCR is the efficient approach for quantitative gene expression study as Liu et al., (2013) also employed the use of qRT-PCR to analyse the wheat yellow vein mosaic virus in wheat. The qPCR method also is a well adopted technique to measure the relative mRNA expression levels in rice plants at 14, 20, 30, 40, and 50 days against southern rice black-streaked dwarf virus (He et al., 2013).

The expression of begomovirus specific coat protein gene was estimated in both resistant and susceptible okra species at 0, 1, 3, 5, 10, 15, 20, 25 dpi through qRT-PCR. The leaf samples were collected for RNA extraction to estimate virus expression as the disease symptoms in the form of yellow veins mostly appear on the leaves. This is the first study to estimate the virus titer at different dpi through qRT-PCR assay in okra. The PCR reaction performed on the normalised cDNA using tubulin gene and begomovirus specific coat protein gene showed amplification of 100 bp and 137 bp respectively. Reactions performed without template yielded no product.

The expression levels of begomovirus coat protein gene were observed in resistant and susceptible species at 0, 1, 3 and 5 dpi. In resistant species, high relative virus gene expression with 1.59 fold change was observed at 5 dpi rather than 0.95, 0.65, 0.44, 0.17, 0.15 and 0.08 fold change at 25, 20, 15, 10, 1 and 3 dpi respectively (Fig. 4 A). Likewise, in susceptible species high relative virus gene expression was 3.51 fold change at 5dpi rather than 1.31, 1.26, 1.02, 0.97, 0.52 and 0.35 fold change that was observed at 10, 25, 1, 3, 15 and 20 dpi (Fig. 4 B). The comparative analysis of gene expression in between resistant and susceptible species showed that virus gene expression higher at 5 and 25 dpi in both species. The begomovirus specific coat protein genes are expressed at much higher rate at 5 dpi that allow the resistant species to resist the attack instead of virus inoculations on the resistant species. The fold change gene expression of cDNA samples of resistant and susceptible species using reference (tubulin gene) and target (begomovirus specific coat protein gene) was analysed through SAS software (https://www.sas.com/).

We found that the use of begomospecific primers in qRT-PCR showed higher virus gene expression at 5 and 25 dpi in the resistant species. We confer that this happens because at these data time points resistant plant resist the attack despite of virus inoculations on the resistant plant. It has also been reported that the high expression level at 5 dpi was observed in response to southern rice black-streaked dwarf virus in different hosts that may indicates that virus enhanced its replication against the strong immune response of plant system (He et al., 2013). We suggest that the expression study at 5 dpi is strong enough to restrict the viral attack in resistant plants. Similarly, the defense response in the resistant hop cultivar is strong enough at 20 dpi to restrict Verticillium wilt fungal disease (Cregeen et al., 2015). Even today, it has been reported that qRT-PCR is preferable method for

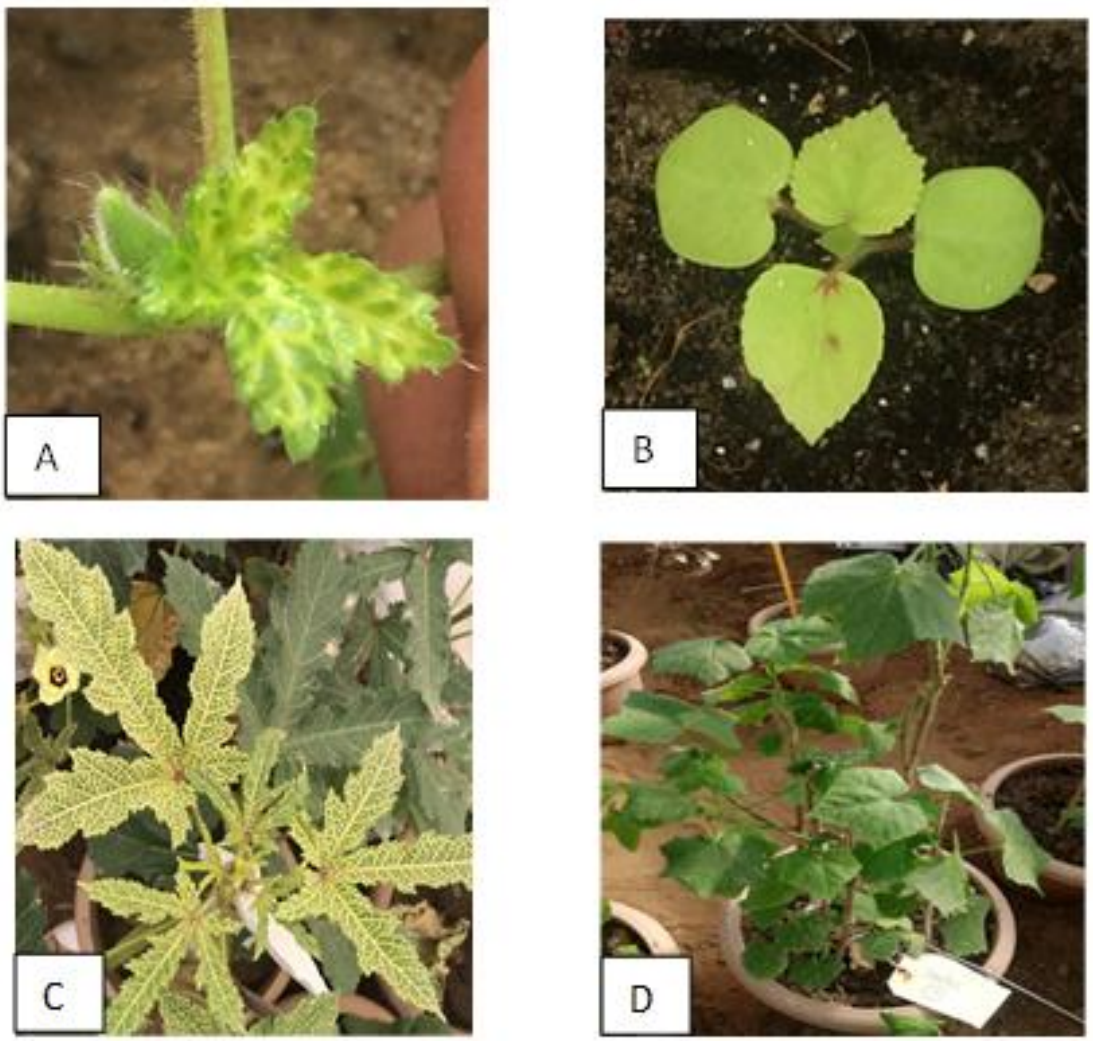


**Fig. 2. Disease symptoms of *Abelmoschus esculentus* cv. Punjab Padmini and *Abelmoschus moschatus* acc 140986 at10 dpi (A, C) and 30 dpi (B, D). *Abelmoschus moschatus* accession 140986 tend to remain as stay green phenotype at 10 dpi and showed no YVMV symptoms even upto 30 dpi while *Abelmoschus esculentus* cv. Punjab Padmini showed YVMV symptoms at both 10 dpi and 30 dpi**

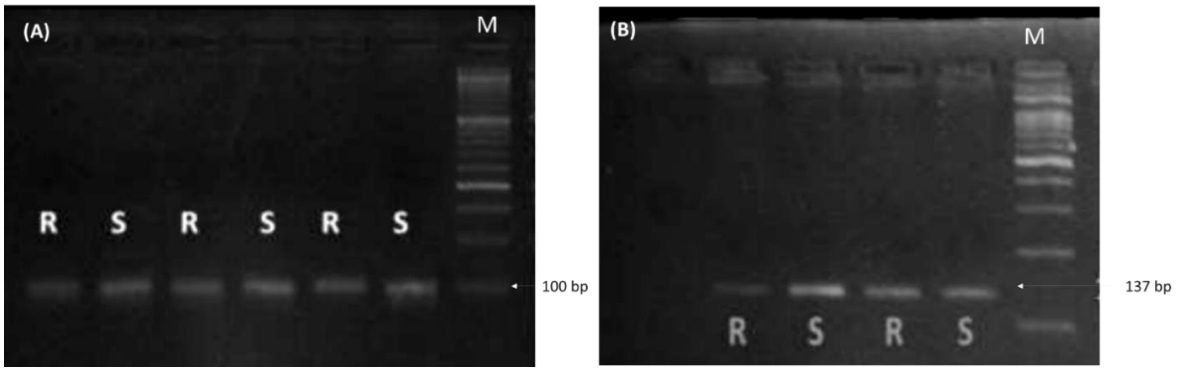


**Fig. 3. cDNA amplification using (A) Housekeeping (tubulin) primer (100 bp) (B) Target (begomovirus coat protein specific) primer (137 bp), R- Resistant, S- Susceptible, M- 100bp marker**

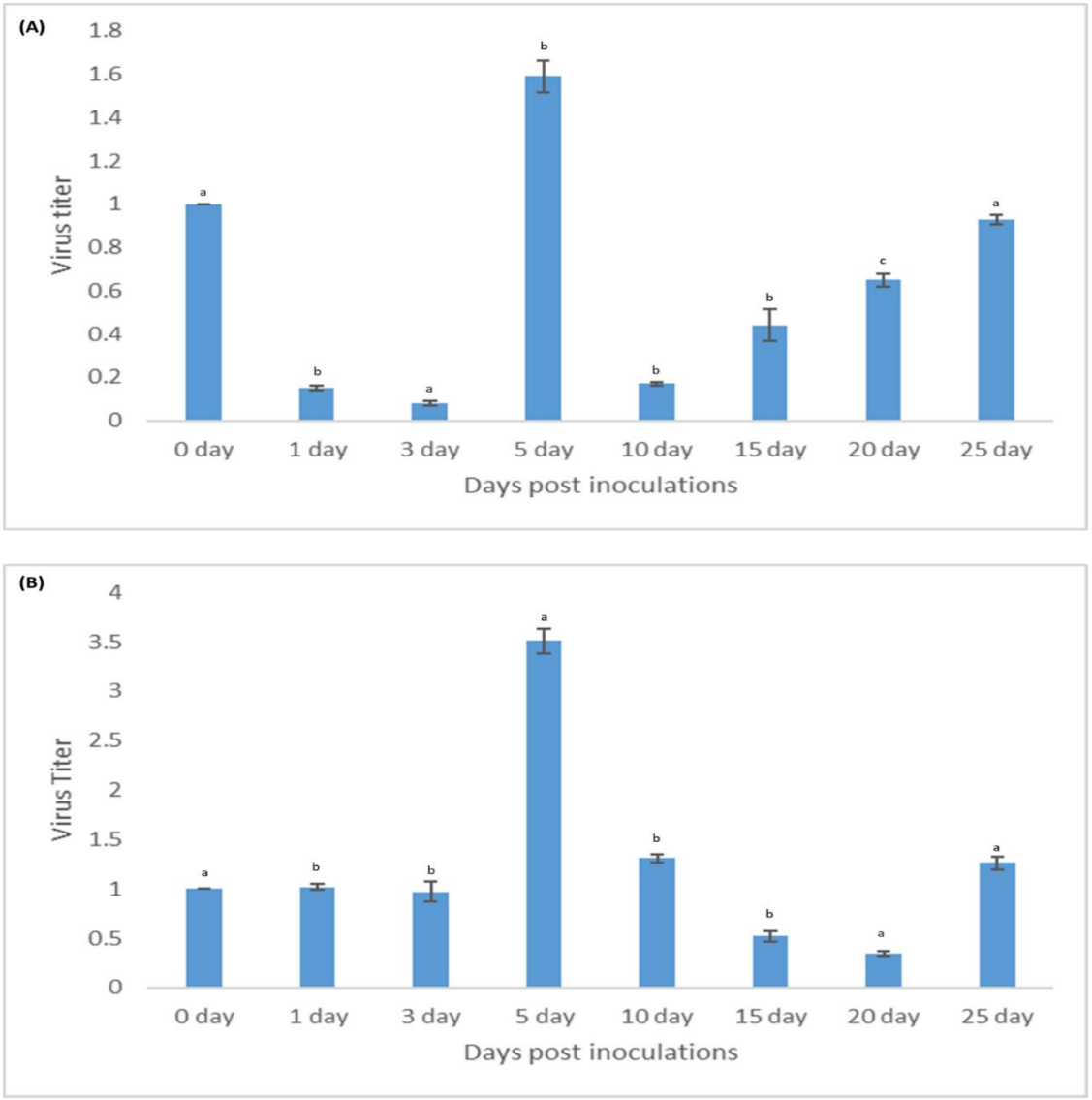


**Fig. 4. Fold change expression analysis in (A) Resistant and (B) Susceptible in three replications showing high virus titer at 5 dpi in both species with respect to resistant (control) and susceptible (control), Duncan's multiple range test (DMRT) analysis of the data shows the mean ± standard error of three independent replications, on the vertical bars different superscript letter is showing the statistically significant difference at p < 0.05**

gene expression profiling that ultimately provide insights into biological systems in tomato lines against geminivirus tomato curly stunt virus (Bokhale et al., 2023). The systemic time virus infection till disease appearance (yellow spots or stripes) on the uninoculated leaves is around 5–11 days post inoculation against Barley stripe mosaic virus in wheat (Lee et al., 2015). The screening of initial time frame from inoculation till the disease appearance in response to disease infection is the stepping stone towards the study of virus resistance mechanism in crops.

## 4. CONCLUSION

We found the statistically based fold change gene expression was higher at 5dpi in both resistant and susceptible species. The expression analysis signifies that virus titer enhanced at 5 dpi in resistant species which would be the considerable time for disease screening with scrutiny. This is the first report suggesting the time frame to explore the virus resistance related genes against YVMV at the molecular level. We cofound that resistance species initiate the onset of resistance genes to

suppress the virus disease attack that fits with the flor-flor hypothesis. Though the disease symptoms appear on susceptible species yet these symptoms do not have any relation with virus titer observed through quantitative study. The obtained results form the basis for further studies on the virus infection and interaction mechanism between YVMV and okra. qRT-PCR represents the true picture of begomovirus specific coat protein gene expression that could be used for transcriptome analysis studies in future. Therefore, we strongly suggest that the temporal gene expression study is important and should be the preliminary approach to decide the exact time interval in response to viral disease.

## DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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