

## Morphological Characterization and Identification of Begomoviruses in Progeny to Row Trials of Okra (*Abelmoschus esculentus* L.)

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**Abstract:** Ninety five okra genotypes were evaluated for morphological differences and yellow vein mosaic virus (YVMV) occurrence in progeny to row trials. Principal component analysis (PCA) was executed to obtain more reliable information. A high degree of diversity was found among okra genotypes for qualitative and quantitative characters. Distance of each variable with respect to PC1 and PC2 showed the contribution of this variable in the variation of germplasm. PCA analysis showed that inter-nodal distance, plant height at maturity, days to first flowering and 100-seed weight are the most important descriptors for variation. For the identification of Begomovirus, the putative viruses were cloned in plasmid and the respective plasmids were restricted by appropriate restriction enzyme. The phylogenetic analysis established that the partial DNA-A resemble to the CLCuMuV-[PK:Fai:Okra:13]. Which is actually a cotton infecting virus and closely resemble to the BYVMV-[IN:Har:05].

**Key words:** Begomovirus, okra, principle component analysis, variability, YVMV.

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### 1. Introduction

Okra (*Abelmoschus esculentus* L.) called bhendi torri in India and Pakistan. It is a vegetable crop grown in the tropical and subtropical zones. It belongs to the family Malvaceae. The production of okra in Pakistan was more than 102600 tons from an area of 13900 ha during 2013 (Anonymous, 2013). In Pakistan, average yield of okra is too low and the lack of high yielding varieties is reckoned to be a crucial cause of the breach in production and demand. Therefore, it is needed to develop high yielding varieties of okra.

Okra is native to Asia, South East States of USA and Middle East. Okra is used for many purposes, as an ingredient for stews and soups (Sengkhamparnet al., 2009) and treatment of dental diseases as a diuretic agent (Ndjouenkeu, 1996). It was also claimed that water extracted polysaccharides from okra might also be used as egg-white substitute (Costantino and Cerpoviez, 2004). The stem of okra could be used as fuel. Okra when grow in early season, has a longer duration, more vigorous and early flowering. (Tremiren and okly, 1986). The yield and vegetative growth could be enhanced by continuous harvesting of immature pods.

Okra is attacked by different diseases incited by fungi, bacteria and viruses. Yellow vein mosaic virus (YVMV) is an economically important viral disease in okra. Bhendi yellow vein mosaic virus (BYVMV) was first reported from Bombay in India (Kulkarni, 1924). Bombay yellow vein mosaic virus (BYVMV) shows

morphological and serological relationship with African cassava mosaic virus (Harrison et al., 1991). YVMV disease was characterized by the formation of yellow patches in the green island of the leaf. In the initial stages, veins become yellow and leaf show creamy or dark yellow color (Ali et al., 2005). YVMV belonged to family Geminiviridae, effect large family of fiber and food crops. Geminiviruses divided into seven genera, viz., Mastrevirus, Curtovirus, Begomovirus and Topocovirus, Becurtovirus, Eragrovirus and Turncurto virus respectively (Nang et al., 2012).

YVMV is a monopartite (DNA A) old world virus (Muniyappa et al., 2003). The monopartite molecule allied of  $\beta$  satellite (1.4 kb) cause typical disease symptoms in leaf. DNA A resides AC1, AC2, AC3 and AC4 genes, which are collectively responsible for replication (rep), synthesis of transcriptional activation protein (TrAP) and replication enhancer (REN) while the AC4 function is still mysterious. TrAP protein for controlling late gene expression, AV1 for pre-coat protein and AV2 for coat protein. The DNA B consists of nuclear shuttle protein (NSP) for nucleus to cytoplasm movement and movement protein (MP) for cell to cell movement (Briddon et al., 2003). In addition to DNA-A, some members of its family associated with DNA- $\beta$  for developing typical disease symptoms. The  $\beta$  satellite has correlated sequence of helper virus, which helps for replication, insect transmission and movement within plants (Saunders et al., 2000).

The first objective was to study the variability regarding different morphological traits among 95 genotypes. The second objective of this research was to make the clones of Begomovirus (2.8kb) and  $\beta$  satellites (1.4kb) using standard protocols. Phi29, which is a substitute of PCR (Polymerase Chain Reaction) due to high processivity and biases, was used to find out the viral load in genomic DNA (Daudet al., 2009). The viral DNA A and  $\beta$ satellite clones were formed to endorse the results.

## 2. Material and Methods

### 2.1 Morphological characterization of Okra genotypes

The research work was carried out in the experimental area of Department of Plant Breeding and Genetics, University of Agriculture, Faisalabad during crop season 2012. Ninety five plant to row progenies were studied under progeny row trials. The spacing level was 40 cm between rows and 20 cm between plants in a randomized complete block design. There were three replications of each okra genotype. Uniform cultural practices and necessary inputs were applied to all the entries in the experiment. The data for the following quantitative characters plant height at maturity, plant height at first flowering, number of nodes at first flowering, days to first flowering, internodal distance, number of primary branches per plant, leaf length, leaf breadth, number of leaves at first flowering, fruit length, fruit diameter, number of fruits/plant, average fruit weight, fruit yield/plant and 100-seeds weight were recorded.

### 2.2 Statistical Analysis

The data obtained for various characters were analyzed through analysis of variance technique to test the significance of differences among various genotypes (Steel et al., 1997).

Principal component analysis (PCA) technique was applied to observe interrelationships among variables (Broschat, 1979). PCA was performed to obtain more reliable information on how to identify groups of genotypes that had desirable yield traits for breeding. PCA can be performed on two types of data matrices, a variance –covariance matrix and a correlation matrix. With characters of different scales, a correlation matrix standardizing the original data set was preferred.

### 2.3 Molecular Characterization of Begomoviruses

#### 2.3.1 Sample Collection

The sample of YVMV infected leaves were collected from the field in polythene bags and placed at -80°C till use.

#### 2.3.2 Using RCA and Restriction Technique

The DNA was isolated from OYVMV infected okra leaves using modified CTAB method (Singh and Kumar, 2012). The DNA of the symptomatic plants was amplified by standard rolling circle amplification (RCA) method using  $\Phi$ 29 polymerase and phi mix (Dean et al., 2001). Then resultant product was run on 1% agarose gel to confirm the amplification along with a 1kb ladder as a standard.

The amplified RCA product was digested with a set of available enzymes producing sticky ends. Restricted fragments of ~ 2.8kb and 1.4kb; DNA-A and satellites respectively, were cloned in pTZ57R/T, having Ampicillin as a selectable marker. The selected positive clones were sequenced to confirm their nucleotide alignment with known viral DNA.

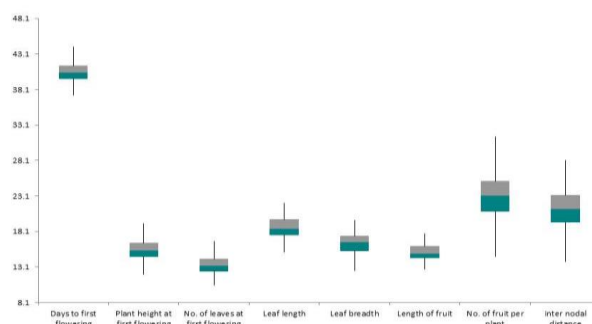
## 3. Results

The data were collected on the basis of quantitative traits show significant difference as shown (Table 1).

**Table 1: Analysis of variance for different plant characters**

Traits	Source of Variation		
	PRTs (94)	Replicates (2)	Error (188)
Days to first flowering	3.51**	4.50 <sup>NS</sup>	1.85
Plant height (1 <sup>st</sup> flowering)	12.67**	10.86 <sup>NS</sup>	7.09
Leaves (1 <sup>st</sup> flowering)	6.03**	1.78 <sup>NS</sup>	2.96
Nodes (1 <sup>st</sup> flowering)	0.18**	0.11 <sup>NS</sup>	0.08
Plant height (maturity)	165.08**	53.74**	99.90
Leaf length	6.47**	2.49 <sup>NS</sup>	2.37
Leaf breadth	6.20**	3.62 <sup>NS</sup>	3.88
Length of fruit	3.82**	1.80 <sup>NS</sup>	1.43
Diameter of fruit	0.25**	0.15 <sup>NS</sup>	0.11
Number of fruits plant <sup>-1</sup>	27.37**	8.07 <sup>NS</sup>	13.93
Average fruit weight	1.09**	0.91 <sup>NS</sup>	0.62
Fruit yield plant <sup>-1</sup>	3618.5**	854.17**	1552.4
100-seed weight	0.33**	0.17**	0.19
Primary branches plant <sup>-1</sup>	0.09**	0.05 <sup>NS</sup>	0.05
Inter-nodal distance	28.46**	10.09 <sup>NS</sup>	12.97

\*, \*\*: Significant at 5%, and 1%, NS: non-significant



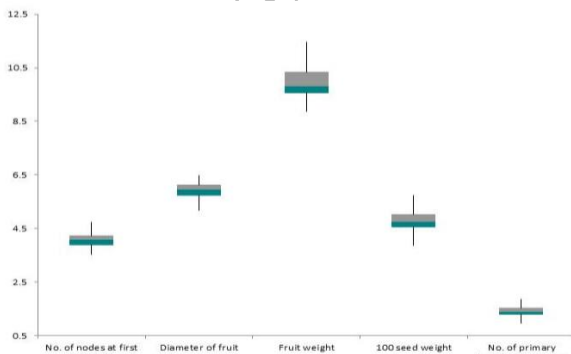
**Fig. 1:** Box Whisker plot for days to first flowering, plant height at first flowering, number of leaves at first flowering, leaf length, leaf breadth, length of the fruit, number of fruit per plant and inter-nodal distance.

PCA was executed to obtain more reliable information on how to identify groups of genotypes that have desirable yield traits for breeding. A 15 × 95 data matrix was performed for the analysis.

**Table 2: Percentage of explained and cumulative variances and eigen vectors on principal components in 95 plant to row progenies.**

No.	Eigenvalue	% Total variance	Cumulative - Eigenvalue	Cumulative - %
1	2.87	19.17	2.87	19.21
2	2.19	14.63	5.07	33.80
3	1.87	12.52	6.94	46.33
4	1.21	8.13	8.16	54.46
5	1.12	7.46	9.28	61.93
6	1.02	6.82	10.31	68.76
7	0.89	5.99	11.21	74.76
8	0.85	5.68	12.06	80.44
9	0.72	4.82	12.79	85.27
10	0.62	4.13	13.41	89.41
11	0.52	3.53	13.94	92.94
12	0.40	2.72	14.34	95.66
13	0.35	2.35	14.70	98.01
14	0.29	1.96	14.99	99.98
15	0.00	0.014	15.0	100.0

Out of fifteen, first six PCS exhibited more than one eigen value but the level of dissimilarity was low which indicated that progenies have narrow genetic base for the traits under study. In the first principal components, plant height at first flowering, number of fruits per plant and days to first flowering were the most important traits contributing towards variation that obtain about 19.175% (Table 2).

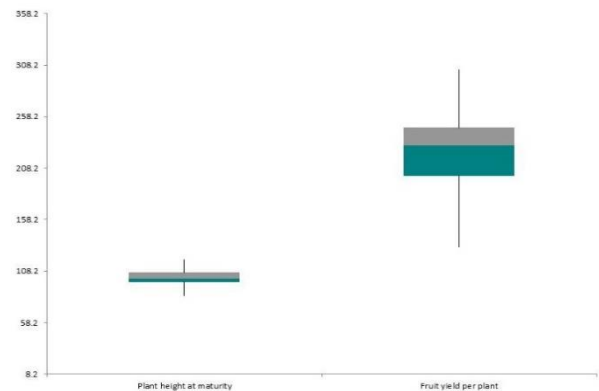


**Fig. 2: Box Whisker plot for number of nodes at first flowering, diameter of fruit, average fruit weight, 100-seed weight and number of primary branches per plant.**

The days to first flowering and number of fruits per plant could be adequate to introduce the difference among plant to row progenies (Table 3). In the second principal component, obtained variation was about 33.80% (Table 2). The intermodal distance, leaf breadth and leaf length were the important trait contributing to variation (Table 1). In the third principal component, days to first flowering, number of leaves at first flowering and number of nodes at first flowering were contributing parameters for variation about 46.33% (Table 2; Table 3).

**Table 3: Principal Components (PCS) for different plant characters.**

Traits	PC1	PC 2	PC 3	PC 4	PC 5	PC 6
No. of primary branches/plant	-0.37	-0.21	-0.25	0.03	-0.02	<b>0.34</b>
Inter-nodal distance	-0.03	<b>0.55</b>	-0.20	<b>-0.49</b>	-0.17	<b>-0.38</b>
Days to 1 <sup>st</sup> flowering	<b>0.37</b>	-0.32	<b>-0.59</b>	-0.19	-0.05	-0.17
Plant height (1 <sup>st</sup> flowering)	<b>0.69</b>	0.25	-0.27	-0.16	0.22	0.02
No. of leaves (1 <sup>st</sup> flowering)	-0.29	-0.33	<b>-0.64</b>	0.12	0.06	0.21
No. of nodes (1 <sup>st</sup> flowering)	-0.29	-0.40	<b>-0.61</b>	-0.07	0.10	0.08
Plant height at maturity	-0.10	0.24	-0.15	-0.43	<b>-0.39</b>	<b>0.42</b>
Leaf length	-0.27	<b>-0.63</b>	0.42	-0.13	0.07	-0.12
Leaf breadth	-0.08	<b>-0.71</b>	0.04	-0.12	-0.24	-0.20
Length of fruit	-0.11	-0.42	0.18	<b>-0.53</b>	0.05	-0.29
Fruit Diameter	-0.37	-0.04	0.38	0.03	<b>-0.51</b>	0.24
No. of fruit plant <sup>-1</sup>	<b>-0.80</b>	0.33	-0.22	-0.04	-0.13	-0.20
Average fruit weight	-0.51	0.08	0.19	-0.08	<b>0.66</b>	0.08
Fruit yield plant <sup>-1</sup>	-0.87	0.33	-0.10	-0.06	0.14	-0.13
100-Seed weight	-0.13	0.02	-0.22	<b>0.61</b>	-0.23	<b>-0.46</b>



**Fig. 3: Box Whisker plot for plant height at maturity and fruit yield plant<sup>-1</sup>.**

In fourth PCA, there was 54.46% variation, which was more related to 100 seed weight, length of fruit and inter-nodal distance (Table 2; Table 3). The progenies were poor for fruit weight, number of nodes



Fig. 4: Leaves of okra plant exhibiting symptoms of OYVMD.

per plant (Table 3). It had negative impact on yield. From first four PCs, it was cleared that among entire fifteen variables number of fruits per plant had high value, while number of primary branches per plant had lowest value (Table 3). PC5 showed 61.93% variation, mainly caused by average fruit weight, plant height at maturity and diameter of fruit were the important characters contributing towards variation. (Table 2; Table 3). PC6 showed 68.76% variation, mainly caused by 100 seeds weight, plant height at maturity, number of primary branches per plant and inter-nodal distance (Table 3).

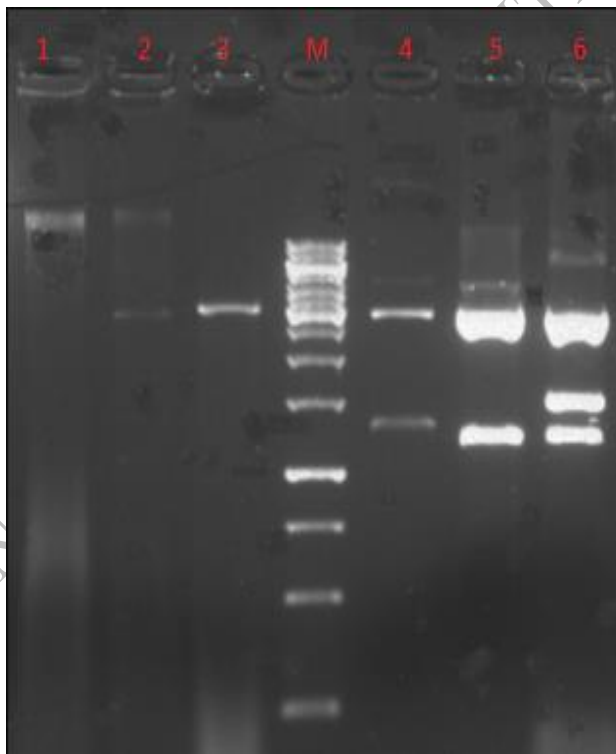


Fig. 5: Show RCA concatamers, 1; restriction of concatamer with HindIII, 2; pTZ57R/T vector, 3; 1kb ladder, M, 4,5,6 are the respective clones of  $\beta$  (1.4kb),  $\alpha$  (1.3kb) and DNA-A (2.8kb).

### 3.1 Molecular Characterization of Begomoviruses

Viruses modulate their genetic make-up according to the prevailing challenges. Virus's lifestyle varies from family to family and virus to virus but the chance of recombination between or within viruses does not follow a hard and fast rule.

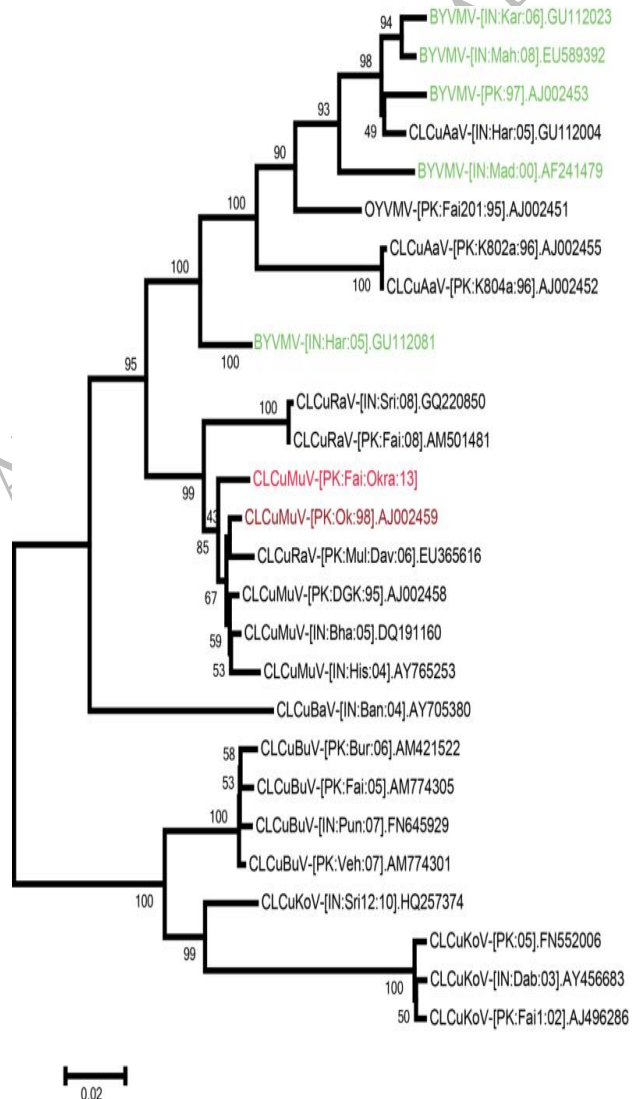


Fig. 6: The sequences of viruses associated with CLCuD and BYVMD were downloaded from NCBI. Their accession numbers are mentioned at the end of each sequence. The newly sequenced molecule (CLCuMuV-[PK:Fai:Okra:13]) was aligned with other representative molecules in Mega5 program. The alignment was constructed through Clustal-W. By using the Neighbor joint method in Mega5 program, the phylogenetic tree was constructed by using 1000 bootstrap replicates. The



acronyms of the viruses were proposed according to ICTV guidelines.

### 3.2 Detection of Viral Molecule by Rolling Circle Amplification

The RCA method was used for the detection of viral molecules in the genomic DNA. RCA is more efficient than PCR due to high processivity. The amplification of DNA by RCA indicated that circular molecules were present. The RCA product was subjected to restriction digestion with appropriate enzyme HindIII released fragment of 2.8kb and 1.4kb (Fig. 5).

### 3.3 Cloning of Restricted Fragment

The respective restricted fragment was treated with phenol chloroform (1:1) and checked by running on 0.5% agarose gel. The restricted fragment was cloned into the respective HindIII site of the pTZ57R. Ligation was good 6:1 (insert:vector). Ligated samples were transformed into *E. coli* top 10 cells. The white colonies were picked and grow in liquid LB (Broth) media at 37°C. The plasmid was isolated by alkaline lysis method (Bimboim and Doly, 1979), followed by double digestion with HindIII and SacI. The fragments of 0.7kb and 2.1kb were released from PTZ vector. The 1.4 kb fragment was also ligated in PTZ57R and confirmed by single restriction by HindIII (Fig. 5). Through sequencing of the selected clones it was found that the CLCuMuV is the causative agent of disease and it is closely related to the BYVMV as show in Fig. 6. This study would be helpful in selection against OYVMD in progeny rows.

## 4. Discussion

The difference among individuals of any population is indication of variability (Poehlman and Sleper, 1995). The existence of variability determines the success of genetic improvement for specific traits (Sharma et al., 2003). To deploy existing variability effectively, it is impressive to characterize the germplasm for morphological traits precisely.

Analysis of variance (ANOVA) specified that there was significant variation due to genotypes for all traits (Table 1). The range of coefficient of variation was 3.36 to 17.32% with maximum value for inter-nodal distance, plant height at first flowering, number of leaves at first flowering, leaf breadth, number of fruits/plant and fruit yield/plant. The days to first flowering ranged from 37.26 to 44.08 days, number of leaves at first flowering ranged from 10.50 to 17.39 and plant height at maturity was 82.14 to 119.3 cm etc.

It was reported that coefficient of variability ranged from 4.58 to 85.48% for various characters maximum value for leaf length, days to maturity, root length and root diameter (Kumar et al., 2011).

The output of PCA revealed that different traits contributed differently to the variation. These differences indicated the present of variability and considerable opportunity for improvement of different qualitative and quantitative traits. The first PC accounts for 19.17% of total multivariate variation, while second 14.63% and third PC 12.52%. The cumulative variation was 46.33%. The high degree of variation 68.76% was observed in first six PC axes. There was no guideline to determine the significance of eigen vectors (Duzyaman, 2005). The higher coefficients for traits substantiated the relatedness of that trait with respective PC axes (Broschat, 1979). The first six components were retained in analysis because eigen values are >1. The other factors having eigen value <1 were ignored. These were ignored due to Guttens lower bound principle, according to which eigen values <1 should be ignored (Kumar et al., 2011).

The first PC axes separated days to first flowering, plant height at first flowering and number of fruits per plant, while second PC axis separated leaf length, leaf breadth and inter-nodal distance. The days to first flowering, number of leaves at first flowering and number of nodes at first flowering were separated in PC3. The fourth PC axis separated inter-nodal distance, length of fruit and 100-seed weight. The PC5 parted plant height at maturity, diameter of fruit and average fruit weight. Early fallouts depicted that various cultivars could be distinguished by their differences in number of pods and pod length, which had the highest coefficient in first PC axes (Vural et al., 2000; Duzyaman and Vural, 2002). Dash (1997) reported that plant height at maturity, days to flowering and seeds per fruit were the highest contributors to variation among okra cultivars. These observations confirmed the individual contribution of these traits to the variation. This was according to the report of Ariyo and Odulaja (1991).

Principal component analysis (PCA) proved to be a better tool which provided genetic variability among okra progenies. From the current study, it was clear that a virtuous breeding programme can be commenced by the selection of progenies from the PC1, PC2 and PC3 those were in total agreement with earlier reports for cowpea (*Vigna unguiculata* L.) and yam (*Ipomoea batatas* L.) (Aremuet et al., 2007).

The phylogenetic analysis was made from partial sequence of DNA-A component by MEGA 5.10 and other sequences get from the available NCBI database as shown in Fig. 6. The phylogenetic analysis demonstrated that the partial DNA-A resemble to the CLCuMuV-[PK:Fai:Okra:13]. Which was actually a cotton infecting virus and closely resemble to the BYVMV-[IN:Har:05]. The CLCuMuV-[PK:Fai:Okra:13] showed 95% identity with BYVMV-[IN:Har:05] (Fig. 6). It was demonstrated that leaf curl affecting okra had virtually the same sequences as those from cotton. The OYVMD distinct but closely related to the virus isolates from cotton (Zhou et al., 1998). The Begomoviruses which effect cotton was founded in okra showing mosaic like symptoms. The CLCuV-PK was known to cause leaf curl symptoms when transmitted experimentally to okra (Harrison et al., 1997). The partial sequence analysis will be helpful to understand the association of different Begomoviruses which infect both cotton and okra.

## Conclusion

The inter-nodal distance, plant height at maturity, days to first flowering and 100-seed weight should be considered in okra improvement program. The number of fruits per plant had direct effect on fruit yield and indirect effect on diameter of fruit. Hence, number of fruits per plant should be considered as major determiner of yield. The presence of different Begomoviruses on okra is a distressing situation and integrated resistant source will be inevitable to sustain okra productivity.

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## Competing Interests

Authors declare that they have no competing interests and commercial names and details of machines and equipments are for the guidelines only.

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