BIOCHEMICAL RESPONSE OF OKRA TO *Bhendi yellow vein mosaic virus* AND PRODUCTION OF VIRUS FREE PLANTS

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by

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THESIS

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DECLARATION

I, hereby declare that the thesis entitled "**Biochemical response of okra to *Bhendi yellow vein mosaic virus* and production of virus free plants**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associate ship, fellowship or either similar title, of any other University or society.

Place : Vellayani

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Certified that this thesis entitled "**Biochemical response of okra to *Bhendi yellow vein mosaic virus* and production of virus free plants**" is a record of research work done independently by Ms. **Swetha B. Nair** under my guidance and supervision and that it has not previously formed the basis of award of any degree, diploma, fellowship, or associateship to her.

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Swetha B Nair

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LIST OF ABBREVIATIONS AND SYMBOLS USED

%	Per cent
µg	Microgram
µl	Microlitre
AEU	Agro ecological unit
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BYVMD	Bhendi yellow vein mosaic disease
BYVMV	Bhendi yellow vein mosaic virus
CTAB	Cetyl trimethyl ammonium bromide
DAG	Days after grafting
DAS	Days after sowing
DAS ELISA	Double Antibody Sandwich ELISA
DIBA	Dot Immuno Binding Assay
DNA	Deoxyribonucleic acid
dsDNA	Double Stranded DNA
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
et al.	And other co-workers
GA_3	Gibberellic acid
ha	Hectare
IAA	Indole acetic acid
KAU	Kerala Agricultural University

mg	Milligram
ml	Millilitre
MS media	Murashige Skoog media
NAA	Naphthalene acetic acid
NCM	Nitro cellulose membrane
OD	Optical density
PAL	Phenylalanine ammonia lyase
PCR	Polymerase chain reaction
PO	Peroxidase
PPO	Polyphenol oxidase
PVP	Poly vinyl pyrrolidone
rpm	Revolutions per minute
SDM	Spray dried milk
sp.	Species
spp.	Several species
TAE	Tris Acetate EDTA
TE buffer	Tris EDTA buffer
ToLCNDV	Tomato leaf curl New Delhi virus
VI	Vulnerability Index
mg	Milligram

Introduction

1. INTRODUCTION

Viral diseases are one of the major threats to sustainable and productive agriculture, resulting in several billion dollars every year. *Geminiviridae* is a diverse family of plant viruses comprising 14 virus genera that cause serious global loss by infecting a wide range of food and fiber crops. The largest genus in *Geminiviridae* is *Begomovirus* with 445 species out of 2244 plant virus species listed (Walker *et al.*, 2022).

Okra [*Abelmoschus esculentus* (L.) Moench] of *malvaceae* family also known as ladies finger or bhindi is one of the most important vegetable crops in the world which is widely cultivated in many tropical, subtropical and warmer parts of temperate Asian countries (Ali *et al*., 2012). Globally, okra is occupying an area of 2.53 million ha, yielding 10.55 million metric tons (MT) annually having an average yield of 4.16 MT ha^{-1}. India ranks first in the world with a production of 6.37 million MT from over 0.5 million ha area with 12.2 MT ha^{-1} productivity (FAO, 2020).

Pest and diseases are a major threat for cultivation of okra. Various bacterial, fungal and viral pathogens cause severe crop loss and yield reduction in okra production. Okra is susceptible to more than 27 begomoviruses due to the warm tropical climate supporting almost round the year survival of the whitefly vector, their overlapping host range, mixed cropping system and polyphagous nature of white fly. Bhendi yellow vein mosaic virus, okra yellow vein mosaic virus, leaf curl virus and Okra enation leaf curl virus are the major viruses of okra (Venkataravanappa *et al.,* 2013). One of the major disease, Yellow vein mosaic disease (YVMD) of okra caused by *Bhendi Yellow Vein Mosaic Virus* (BYVMV) was first reported in 1924 from India (Kulkarni, 1924).

Bhendi yellow vein mosaic virus belongs to *Monodnaviria* (Realm); *Shotokuvirae(*kingdom*);Cressdnaviricota* (phylum);*Repensiviricetes* (class);*Gepla fuvirales* (order); *Geminiviridae (*family*); begomovirus (*genus).

Based on their host range, genome organization and insect vectors affecting

them, *Geminiviridae* are classified into 14 genera *viz.*, *Becurtovirus*, *Begomovirus*, *Capulavirus*, *Citlodavirus*, *Curtovirus*, *Eragrovirus*, *Grablovirus*, *Maldovirus*, *Mastrevirus*, *Mulcrilevirus*, *Opunvirus*, *Topilevirus*, *Topocuvirus* and *Turncurtovirus* (ICTV reports, 2022). The majority of begomoviruses have a genome comprising two similar sized DNA components (DNA A and DNA B). The DNA A component encodes a replication-associated protein (Rep) that is essential for viral DNA replication, a replication enhancer protein (REn), the coat protein (CP) and a transcription activator protein (TrAP) that controls late gene expression. The DNA B component encodes a nuclear shuttle protein (NSP) and a movement protein (MP), both of which are essential for systemic infection of plants (Hanley-Bowdoin *et al*., 1999; Gafni and Epel, 2002).

Vector of bhendi yellow vein mosaic disease is whitefly, *Bemisia tabaci* which belongs to *Aleyrodidae* family of order *hemiptera*. *B. tabaci* is a complex species having more than 30 morphologically indistinguishable cryptic species (De Barro *et al*. 2011; Boykin *et al*. 2013). Among them, the two most invasive and destructive species are the Middle East-Asia Minor I species (MEAM-1), and the Mediterranean species (MED) formerly referred to as biotype B and Q, respectively (Pan *et al*. 2012).

Curing of viral diseases is not easy as that of fungal and bacterial diseases which can be treated with antibacterial and antifungal compounds. Implementation of appropriate pathosystem (virus, host, and environment) for specific effective viral disease management strategies is an absolute necessity. Chemical treatments had very little success in controlling BYVM disease. Development of host resistance is the only reliable mechanism to manage this disease. Host resistance against the okra yellow vein mosaic disease is the most economical and environment friendly method to increase the yield potential of okra.

Seed transmission studies on BYVMV virus is insufficient. A precise knowledge on the seed transmission is also essential to manage the BYVM disease effectively. Seed infection allows the virus to endure for extended duration in the absence of hosts and/or vectors. Numerous seed-transmitted viruses can persist

within the seed as long as it remains viable. Early infection of disease will result in higher economical damage.

Unstable resistance and limited availability of BYVMD resistant varieties is the major constraint for okra crop improvement programme. Identifing the resistant genotypes and genetic basis of resistance to begomoviruses infecting okra, under different environmental conditions is an absolute necessity to manage the disease. `Different varieties of okra can be screened for disease severity by grafting it with infected scion. In this study, fifteen distinct genotypes of okra were screened for BYVMV resistance under natural epiphytotic conditions of Kerala to determine the possible sources of resistance.

Analysis of BYVMV susceptible and less susceptible genotypes for understanding biochemical mechanism of host resistance is prerequisite for developing an effective disease resistance breeding strategy.

Production of virus free plants can be ensured through meristem culture and maintaining these plants under glass house condition can help in the production of disease-free seeds.

The current study was conducted to establish an appropriate procedure for propagation of okra clones via meristem culture, with the objective of generating potentially virus-free robust plants for the purpose of seed production. Studies on host-pathogen interaction and screening of varieties for disease resistance and development of a technique for rapid multiplication of virus free mericlones can contribute for the development of an effective management strategy for BYVM disease.

Review of Literature

2. REVIEW OF LITERATURE

Okra is one of the top fresh vegetable exports from India, accounting for 70% of all fresh vegetable earnings (APEDA, 2000). Okra contains high quantity of nutritients which are sufficient to improve the nutritional status of malnourished people. It is a good source of dietary fiber, protein, vitamin C, vitamin K, and unsaturated fatty acids (Gemede, *et al.*, 2015). Due to its rich dietary fiber and unique seed protein balance of lysine and tryptophan amino acids, it is regarded as a protective supplementary food.

Okra production is severely affected by various abiotic and biotic factors. BYVM disease is one of the major constraints for okra production. Disease symptoms include yellowing of veins and veinlets, vein thickening, stunting of plant and bleaching of fruits (Venkataravanappa *et al*., 2012). Whitefly (*Bemisia tabaci*) which belongs to *Aleyrodidae* family of order *hemiptera* is the vector of BYVMD and heavy infestations of whiteflies are found in okra plants in field conditions (Venkataravanappa *et al*., 2015).

Pun and Doraiswamy (1999a) observed an economic yield loss of 94- 100 % in okra plants infected at 20 days after sowing whereas Sastry and Singh (1974) observed 49–84% yield loss if the infection occurred 50–65 days after sowing. Fajinmi and Fajinmi (2010) reported a yield loss of 50 - 94% in fields with 100 % disease incidence of BYVMD.

BYVMV is in the largest genus *Begomovirus* under *Geminiviridae* family which include small circular ssDNA viruses with a geminate morphology. The genomes of begomoviruses are composed of either a single component or two components. These components are referred to as monopartite (DNA-A genome component) and bipartite (DNA-A and DNA-B genome components) viruses, respectively (Hanley-Bowdin *et al.*, 1999). Additionally, the monopartite begomoviruses are associated with additional ssDNA molecules known as betasatellites and/or alphasatellites (DNA1). These satellites rely on their helper virus for replication, movement, encapsidation, and vector transmission (Lefeuvre *et al*., 2010).

2.1 COLLECTION AND MAINTENANCE OF VIRUS INFECTED PLANTS

Capoor and Varma (1950) reported that the characteristic symptoms of okra yellow vein mosaic disease are vein clearing, chlorosis, swelling of the veins along with a small downward curling of the leaf edges, twisting of the petioles, and growth retardation in severe cases. Vein clearance and subsequent yellowing were persistent symptoms in okra plants infected with bhendi yellow vein mosaic virus (BYVMV). There is a considerable drop in the amount of vegetable produced, along with smaller leaves and fruits. Reports of yield losses up to 96% have been recorded (Pun and Doraiswamy, 1999b).

According to Swanson and Harrison (1993), okra is known to be vulnerable to at least 27 begomoviruses. Yellow vein mosaic disease (YVMD) and okra enation leaf curl disease (OELCD), have a significant impact on the crop's yield and fruit quality. According to research by Venkataravanappa *et al*. (2013), cultivated okra is primarily vulnerable to a variety of begomoviruses with overlapping host ranges, including radish, tomato, cotton, etc. Okra leaf curl disease (OLCD), okra enation leaf curl disease (OELCD), and yellow vein mosaic disease (YVMD) are caused by viruses of the genus *Begomovirus* which cause significant losses in okra cultivation.

On the axial side of the leaves, yellow vein banding may be followed by interveinal clearing and minute venation (Fernando and Uduravan, 1942). Furthermore, according to Benchasri, S. (2012), the BYVMV infected plants produced a smaller number of leaves and fruits compared to the healthy plants. Infected plants in their early stages, showed stunted growth and development. The fruits of infected plants were small and hard with low-quality which were sometimes with deformed shapes and yellow colour (Jamir *et al*., 2020). Sometimes, the yellow network of veins is followed by thickening of veins and vein-lets (Nariani and Seth, 1958).

Salehuzzaman (1985) studied BYVM disease using bud grafting. Buds collected from the resistant plant were contact grafted on the infected plant and the buds of resistant plant (scion) was contact grafted with susceptible infected stock.

Although they continued to grow on infected plants, these buds later sprouted and matured into complete plants, however in both contact and bud grafting, the resistant plant remained healthy. According to Manjula (1986), side grafting of BYVMV infected scions on to healthy rootstock resulted in 87 to 100 percent disease transmission and inoculated plants started showing BYVMD symptoms 15 to 20 days after grafting. Ali *et al. (*2000) performed grafting trials using resistant plants as rootstock and susceptible plants as the scion whereas Sindhumole (2003) used resistant plant twigs as scion and susceptible plants as the rootstock. Ghevariya and Mahatma (2017) reported approach grafting and veneering as the most effective ways to transmit geminiviruses like BYMV for disease screening. The virus produced typical vein yellowing and thickening of leaves forming a network of veins and veinlets in the infected leaves. Naveen (2016) reported 70 per cent graft transmission of geminivirus in okra. Serological detection studies revealed that BYVMV is closely related to Sri lankan cassava mosaic virus (SLCMV). A vulnerability index (V. I.) of 78.42 and 100% disease incidence were found in the Chirayinkeezhu taluk of Thiruvanavthapuram district (Kerala). Studies conducted by Bincy (2019) have reported 100% graft transmission of bhendi yellow vein mosaic virus.

Prakasha *et al.* (2009) studied okra farms in Karnataka (Dharwad, Haveri, and Bengaluru) and reported the BYVM disease incidence ranges from 0.5 to 48.9%. Evaluated 55 okra genotypes were evaluated for the presence of yellow vein mosaic and found that the disease incidence and coefficient of infection ranged from 7.2 to 100.0 and 1.8 to 75.0 respectively. Senevirathna *et al.* (2016) reported 90 per cent disease incidence of BYVMD with the characteristic symptoms of vein clearing of leaves and bleaching of fruits from Sri Lanka.

Okra samples with BYVMV infection were collected from three fields in Thiruvananthapuram district: Vellayani, Pappanchani, and Palappur. All those fields were severely infested and the okra var Kiran in Palappur had the highest disease incidence (D. I.) of 100% and vulnerability index (V. I.) of 71.07.

Vein clearing, vein thickening, reduced leaf size, stunting, reduced fruits and bleached fruits were major symptoms in surveyed area (Chippy, 2020).

2.2 SEROLOGICAL AND MOLECULAR DETECTION

Clark and Adam (1977) described a microplate method that allows the detection and quantification of plant viruses using an enzyme-labelled antibody. This technique helps to detect presence of numerous morphologically distinct viruses both in purified and unclarified infected plants extracts. Photometric measurement of colour intensity of hydrolyzed substrate using an ELISA plate reader aids the quantification of virus.

For the detection of geminiviruses, particularly begomoviruses, enzyme-linked immunosorbent assay (ELISA) had been treated as an easy and reliable immunological technique (Muniyappa *et al.*,1991; Harrison *et al*., 1991; Swanson *et al.*, 1992). By using an okra yellow vein mosaic virus (OYVMV) specific antiserum and a DAS ELISA, Sheikh *et al*. (2013) were able to identify the presence of *geminivirus* in 86 to 100 samples.

The causative agent of yellow vein mosaic disease, according to Handa and Gupta (1993), is a *geminivirus* with 18 nm to 30 nm in size and closely related to the *Indian cassava mosaic geminivirus* (ICMV), which has been proven by ELISA by employing ICMV polyclonal antiserum. Pun *et al.* (1999) used polyclonal antibodies produced against the African cassava mosaic virus and the Indian cassava mosaic virus to identify OYVMV in infected okra plants using direct antigen coating ELISA (DAC-ELISA).

According to Fauquet and Stanley (2003) the most rapid and sensitive technique for detecting begomoviruses is PCR and the sequence analysis of the coat protein (CP) gene region is adequate for the preliminary detection of the *Begomovirus*. For the detection of begomoviruses such as BYVMV, bittergourd mosaic virus, tomato leaf curl virus, and pumpkin yellow vein mosaic virus, AV/AC primer specific to coat protein of the begomoviruses were successfully used (Naveen, 2016; Bincy, 2019).

A simple but highly reproducible immunodiagnostic technique is the dot-immunobinding assay (DIBA). Antibody will be dotted on nitrocellulose membrane (NCM) discs and the diagnostic material that needs to be examined can be incubated over it. The use of enzyme-conjugated antiglobulin and substrate allows for the direct demonstration of the presence of antigen-antibody complex in NCM discs. The assay will be regarded successful if a purple-pink, insoluble substrate product forms in the NCM (Sumi *et al.*,2009).

In numerous research, enzyme-linked immunosorbent assays (ELISA) were used for serological detection of the okra yellow vein mosaic virus (OYVMV). OYVMV infection was found in symptomatic okra cultivar leaves (Das *et al.*, 2020).

Molecular detection of okra yellow vein virus (OYVV), was achieved using specific primers in several studies. Deng *et al.* (1994) created degenerate primers for the detection of whitefly transmitted geminivirus from the DNA-A of five geminiviruses which yield a PCR product of 500 bp to identify known and possible geminivirus infections in plant species. The presence of OYVV was confirmed in symptomatic okra plants in Sri Lanka using degenerate primers, and the DNA-A genome of the virus was cloned and sequenced (Jeyaseelan *et al.*, 2021).

Venkataravanappa (2018) has done PCR-based begomovirus detection for 340 okra samples, of which 290 samples were associated with monopartite virus and 53 samples were bipartite virus. Genome sequencing of DNA A and B components demonstrated nucleotide identity of 90 per cent with ToLCNDV. According to their phylogenetic and recombination research, DNA A and DNA B components of the ToLCNDV that infects okra have recombinant origins from BYVMV, ToLCNDV, and SLCCNV.

The complete nucleotide sequence of the DNA-A genome of OYVMV has been determined, showing high similarity with mesta yellow vein mosaic virus (MYVMV). A quantitative PCR (qPCR) assay has been developed to detect and quantify the symptom modulating satellite molecules of OYVMV. The genome of

OYVMV has been amplified and sequenced, revealing the association of two different begomovirus species, *Bhendi yellow vein mosaic virus* (BYVMV) and *Okra enation leaf curl virus* (OELCV), with OYVMV in Sri Lanka. Betasatellite DNA sequences associated with OYVMV have been characterized, showing genetic similarity with betasatellites reported from India. PCR analysis using specific primer pairs was used to detect the presence of OYVV in different okra genotypes, with one genotype showing resistance to the virus (Patil *et al.*, 2018; Tharmila *et al*., 2020).

2.3 SEED TRANSMISSION STUDIES

The numerous national and international laws on seed phytosanitary measures indicate that seed transmission is crucial in plant virus epidemics and of substantial concern for food safety (FAO, 2020). This is very important as presently quarantine laws do not exist for the check of movement of any of the *Begomovirus* through seeds.

Seed-transmitted viruses can persist within the seed as long as it remains viable thus seed infection gives the virus a way to persist for extended periods of time when hosts and/or vectors are not available (Sasthri, 2013). Virus seed transmission may provide vital advantages for plant viruses in the era of climate change. It helps viruses to survive unfavorable conditions like prolonged drought periods and facilitate virus geographic range expansion in favorable climatic conditions (Jones, 2016).

Padda (1968) reported BYVMV is readily transmitted through white fly (*Bemisia tabaci*) but neither sap nor seed transmitted.

Sisodia and Mahatma (2020) found that the BYVMV is present in all parts of the okra plant, including seeds, however in a grow-out test, none of the samples displayed symptoms on the seedling. Seed transmission of field bean yellow mosaic disease caused by the *begomovirus*, dolichos yellow mosaic virus (DoYMV) was reported by Suruthi *et al.* (2018). DAS-ELISA and PCR were used to confirm the

presence of DoYMV in mature seeds and 30 day old non-symptomatic grow out plants.

Viruses that spread through seeds have two non-exclusive entry points. The direct entry to embryonic tissue or by infecting the ovules and/or pollen. Plant viruses are confined to the intracellular compartment (symplast) in contrast to animal viruses, which can use the extracellular environment for within-host spread. Viral movement proteins facilitate their intercellular movement through plasmodesmata (Hull, 2014).

Report of mungbean yellow mosaic virus (MYMV) presence in seeds of mungbean (*Vigna radiata* L.) cv GM-4 from Navsari (Gujarat, India), questioned and rejected the concept of non-seed transmission of begomoviruses (Pawar, 2015). Thereafter, reports of the presence of sweet potato leaf curl virus (SPLCV) in sweet potato seed, tomato yellow leaf curl virus (TYLCV) in tomato seed, and mungbean yellow mosaic virus (MYMV) in black gram seed have proved the seed transmission of begomoviruses (Kil *et al.*, 2016; Kim *et al*., 2015; Kothandaraman, 2016).

The presence of Mungbean yellow mosaic virus (begomovirus) in the cotyledon of the *Vigna radiata (*green gram*)* is evident, but there is no report of seed transmission in grow out test. Virus is absent in the embryo and the transmission of virus from seed to seedling is impossible. *Vigna radiata* seeds are epigeal in nature and it results in the emergence of the entire seed including the seed coat from the soil during germination. Whiteflies feed on this succulent cotyledon and acquire the virus. Subsequently, when these whiteflies feed on other seedlings, they transmit the virus. As MYMV does not possess the ability to move from the seed to the seedling on its own and requires a vector, namely the whitefly, for its transmission, the term "indirect seed transmission" was coined to describe this phenomenon (Mahatma and Pawar, 2015).

Kil *et al*., 2016 made an observation that when healthy tomato seedlings are cultivated alongside tomato seedlings that have emerged from infected fallen fruits of tomato leaf curl virus (begomovirus) and non-viruliferous vectors (whiteflies) within a containment structure, the healthy seedlings become infected with the

virus. These infected seedlings also display symptoms, and the virus can be detected in the whiteflies as well. This discovery indicates that the virus is transmitted from the seed to the seedling. However, initially, the presence of the virus does not result in any visible symptoms. It is only after the whiteflies acquire the virus and transmit it to other seedlings that symptoms start to manifest. In contrast, Pérez-Padilla *et al.*, 2020, have refuted this concept in the case of tomato yellow leaf curl virus-Israel (TYLCV-IL). Moreover, the experiment conducted to validate the transmission of the virus from seedlings grown from fallen tomato fruits within the containment structure is deemed inappropriate. However, the presence of TYLCV-IL in tomato seeds including embryo was confirmed, but the seed transmission of virus was not proved in grow out test.

Bincy (2019) reported zero seed transmission of BYVMV in okra by growing ten seeds collected from disease infected plants in insect proof condition for two months.

2.4 SCREENING OF DIFFERENT GENOTYPES OF OKRA BY GRAFT TRANSMISSION

Most practical, economic and environment friendly strategy for reducing viral diseases and yield loss is utilization of host plant resistance (Manjua *et al.*, 2018).

The transmission of resistance from wild relatives is impeded by sterility issues which challenge the production of subsequent generations (Sanwal *et al.*, 2014). The identification of a persistent source of YVMV resistance in okra using genotype screening offers an opportunity for the creation of disease-resistant cultivars. Only one genotype (IIVR11) out of a total of 32 genotypes showed resistant to the YVMV (Kumari *et al.*, 2018).

The Eleventh Workshop of the Project Directorate of Vegetable Research recommended Arka Anamika (Sel-10) for all the regions in India where YVMV is a severe problem (PDVR, 1990). Mathew *et al.* (1993) screened different okra varieties and concluded that Pusa Sawani and AROH-1are highly susceptible whereas in Sel-4 and Arka Anamika, the BYVMD infection was less. Sel-4 and

Arka Anamika are recommended as promising varieties due to high yield and BYVMD resistance.

Srivastava *et al*. (1995) studied the reaction of 12 okra varieties against YVMV in the field at three sites. Varsha Upahar and HRB 55 were free of the disease at Karnal and HY6 at Andhra Pradesh. Arka Anamika showed moderate resistance at Karnal.

Due to the emergence of new virus strains or due to recombination within the virus strain Popular varieties like Parbhani Kranti, Punjab 7, Arka Anamika, and Arka Abhay have lost their YVMV resistance in recent years (Sanwal *et al*., 2014).

Rashid *et al.* (2002) screened 12 germplasm accessions under field conditions and lines OK 292 and OK 285 showing resistance to YVMD were identified. Fairly high degree of tolerance with 10.00 to 21.25 per cent disease incidence was shown by four varieties and other six varieties were highly susceptible with 80-100 per cent disease incidence.

Out of 29 okra genotypes screened under artificial and natural conditions, none of the genotypes showed immunity to BYVM disease. Moderate resistance reactions were shown by Nun 1145 and Nun 1144 (Venkataravanappa *et al*., 2012). Mathew *et al.* (2022) conducted field studies and categorized six genotypes as highly susceptible, 27 genotypes as susceptible and observed Susthira as a resistant genotype against BYMV.

Deshmukh *et. al.* (2011) reported prominent variation in yellow vein mosaic virus incidence due to environmental conditions as it varies from season to season by screening thirty five okra lines. Graft transmission tests were performed for chosen genotypes which showed resistance to yellow vein mosaic disease during the vector transmission studies. The chosen genotypes were used as the root stock in this instance. The infected scion was wedge grafted into the healthy root stock after making a vertical cut on healthy plant and 'V' shaped wedge on scion (Nikitha, 2016).

Two weeks old healthy seedlings of "IPSA Okra 1" (tolerant) were grafted with"SL-44" (susceptible) using the tongue approach grafting technique to study

the transmissibility of YVMV by graft union. "IPSA Okra 1" and "Parbhani Kranti" served the similar function. Keeping the grafted plants in between, seedlings of parental lines were transplanted during both seasons in two distinct plots (Ali *et al*., 2000).

2.5 BIOCHEMICAL ANALYSIS

Several crops show significant difference in biochemical components like phenols and the related enzymes which generate either a susceptible or resistant reaction in host. BYVMD susceptible okra cultivars were inferior to resistant wild okra in terms of seed soluble protein content, phenolics, peroxidase, and polyphenol oxidase activities. Increase in amount of peroxidase and poly phenol oxidase result in higher concentration of phenols and quinones which may have ultimately led to reduced virus multiplication and resistant response in wild okra and its interspecific hybrids (Prabu and Warade, 2009).

In resistant okra cultivars, the phenol, polyphenol oxides, and peroxidase activities contributed to the development of resistance. Quinin can inhibit viral transmission, eventually protecting resistant crops from the disease since it is made up of a lot of phenols and their oxidant products such as peroxide and polyphenol oxides (Mahajan *et al.*, 2004).

In YVMV resistant plants infected with OYVMV, the phenolic content decreased, while the peroxidase and polyphenol oxidase activity, total nitrogen, and sugar content increased when compared to OYVMV resistant healthy plants. Conversely, the opposite trend was observed in OYVMV susceptible healthy and infected plants. The higher concentration of phenols and their oxidation products, such as quinines formed by increased peroxidase and polyphenol oxidase activity, may be responsible for inhibiting virus reproduction. This could ultimately lead to a resistant reaction in wild okra and their interspecific hybrids. Peroxidase is a crucial enzyme involved in the synthesis of lignin. The increased activity of peroxidase has been linked to various physiological functions that may contribute to resistance, including the release of hydroxyl cinnamyl alcohol into free radical

intermediates and lignification (Walter, 1992).

Enzyme activities in okra leaves, including peroxidase and polyphenol oxidase, total phenol and ascorbic acid is negatively correlated with the PDI of the YVMV disease even during different growth stages. This indicates the potential of difference in biochemical response as selection indices for screening genotypes resistant to the YVMV disease (Seth *et al.,* 2017).

Several biochemical parameters and defense related enzymes are associated with OELCuD resistance responses. When developing a selection program for a disease resistance breeding program, it is essential to understand the association between OELCuD susceptibility and biochemical characteristics (Yadav *et al.*, 2020).

A progressive increase in defense related enzymes like peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL) activity is reported in inoculated and susceptible varieties of cowpea (Umamaheswaran, 1996).

Banana bunchy top disease resulted in a notable increase in peroxidase activity within the banana cultivar. Healthy plants are with significantly lower concentration of peroxidase activity compared to banana bunchy top virus *i*nfected plants. Similar findings were observed in tobacco mosaic virus (TMV) infected tobacco plants (Lagrimini and Rothstein, 1987). Geminiviruses like tomato yellow leaf curl virus (TYLCV), banana bunchy top virus (BBTV), chilly leaf curl virus, cotton leaf curl burewala virus (CLCuBuV) infected plants are also showing an increased peroxidase response than the healthy plants (Dieng *et al*., 2011; Devanathan *et al.,* 2005; Meena *et al*., 2000).

The presence of polyphenol oxidases (PPO) enhances plant defense mechanisms. The increase in polyphenol content stimulates phenol production, which effectively limits the spread of the pathogen by generating chlorogenic acid, creating an unfavorable environment for pathogen proliferation (Siddique *et al.*, 2014). Banana bunchy top infection leads to an elevation in PPO activity within banana cultivars. The combination of PPO with phenols activates the plant's defense mechanisms against the pathogen (Ngadze *et al*., 2012).

2.6 PRODUCTION OF VIRUS FREE PLANTS USING MERISTEM CULTURE

Anisuzzaman *et al.* (2008) described protocol for mass *in vitro* propagation of okra using meristem culture. Three-week old shoot tip meristems were isolated from *in vitro* grown seedlings for production of virus free plants for seed production. MS liquid media with 1.0 mg/l of BAP was efficient for growth of isolated meristems. Semisolid MS medium with 1.0 mg/l of BAP was discovered to be the most efficient for shoot development from meristems.

Kabir *et al.* (2008) conducted studies to determine the optimal dose of growth regulators to induce callus and organogenesis in *Abelmoschus esculentus* (L.) Moench. MS media supplemented with 2.0 mg/l NAA and 0.5 mg/l BAP from hypocotyl explant was found to be the best media for callus induction with 95 percent regeneration. More callus was produced by hypocotyl explants.

Compared to native seed-derived plants, meristem-derived plants had greater vigor, higher yields, and healthier seeds (Alam *et al.*,2004). Ganesan *et al.* (2007) have developed a simple and reliable protocol for somatic embryogenesis of okra from suspension cultures. Medium containing half-strength MS salts, B5 vitamins, benzylaminopurine (BAP), and gibberellic acid (GA_3), the highest (67.3%) conversion of embryo into plantlets was observed.

In vitro regeneration of okra (*Abelmoschus esculentus* L.) is challenging because of its recalcitrant nature. Formation of whitish colored "non-embryogenic" callus is a major constraint in okra tissue culture. Different concentrations of inorganic nitrogen (KNO_3 and NH_4NO_3) and plant growth hormone combinations have been tested to produce friable and embryogenic callus masses on MS media. Media containing 1.0 ppm BAP + 1.5 ppm 2, 4-D was observed to be best for maximum callus induction (71%) of Hypocotyl explants. Whereas media containing 1.5 mg/l NAA + 0.5 mg/l BAP is suitable for cotyledon explants (Rizwan *et al.*, 2020).

Hypocotyl, cotyledon, cotyledonary node and leaf segments from aseptically grown okra seedlings were used as explant. Growth hormones like Auxins, cytokinins, and auxin-cytokinin combinations were added to a Murashige and Skoog nutrient medium for explant culture. Naphthalene acetic acid (NAA) or indole acetic

acid (IAA) supplemented media was best for callus development and root differentiation. Higher root proliferation was observed on the medium with NAA but 2,4-Dichlorophenoxyacetic acid (2,4-D) inhibited root development. At any of the auxin levels tested, no shoot bud or shoot development was noticed. Media supplemented with zeatin (Z) and kinetin (KN) was also ineffective for shoot buds or shoot initiation. Cotyledon and cotyledonary node explants grown in medium supplemented with benzyl adenine and NAA showed higher shoot development and root proliferation. Plantlets grew normally when transferred to soil (Mangat and Roy, 1986).

Materials and Methods

3. MATERIALS AND METHODS

The research work entitled 'Biochemical response of okra to *Bhendi yellow vein mosaic virus* and production of virus free plants*'* was carried out at Department of Plant Pathology, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala during the academic year 2021-2023. The objective of this study was to study host pathogen interaction in *Bhendi yellow vein mosaic virus* (BYVMV) infected okra, screening of different genotypes of okra cultivars for disease resistance and production of virus free plants using meristem culture. The materials and methodologies adopted for this research are detailed in this chapter.

3.1 COLLECTION AND MAINTENANCE OF VIRUS INFECTED PLANTS

Purposive sampling survey was done in various locations and symptoms were studied. Virus inoculum was maintained on susceptible host plants.

3.1.1 Collection of BYVMV infected samples and symptomatology

Purposive sampling survey to study symptomatology, disease incidence and vulnerability index of BYVMV was conducted in different locations in Thrissur (AEU 10- North central laterites), Thiruvananthapuram (AEU 8- Southern laterites) and Palakkad (AEU 10- North central laterites) districts. Infected samples of okra were collected from College of Agriculture Vellayani, Thiruvananthapuram **(**plots of Instructional farm, Department of Vegetable science and Department of Soil Science & Agricultural Chemistry), College of Agriculture Vellanikkara, Thrissur and farmers field in Palakkad. Infected leaf samples were stored in -80^0 C deep freezer for further molecular detection and plants were maintained in insect proof glass house. Weeds present in infected fields were also observed and plants with virus disease symptoms were collected.

The variety, characteristic symptoms of disease, disease incidence and vulnerability index were recorded. Different types of symptoms in naturally infected and graft inoculated okra plants were observed and documented.

Vulnerability index (Bos,1982) was calculated according to the 0-5 scale developed by Naveen (2016) (Plate 1).

0 - No symptom

1 - Very mild mottling, initial vein clearing

2 - Mottling and complete yellowing of veins with green interveinal region

3 - Blisters and raised surface on leaves

4 - Distortion of leaves, curling with reduction in leaf size

5 - Stunting with negligible or no flowering and deformed pods

The vulnerability index (V. I.) (Bos, 1982) and disease incidence (D. I.) in each location was calculated using the formulae

$$\text{Vulnerability Index} = \frac{(0n0+1n1+2n2+3n3+4n4+5n5) \times 100}{nt\,(nc-1)}$$

n_0 , n_1, n_3, n_4, n_5 : Number of plants in the category of 0,1,2,3,4, and 5

n_t :Total number of plants

n_c:Number of categories

$$\text{Disease incidence} = \frac{\text{Number of plants infected}}{\text{Total number of plants}} \times 100$$

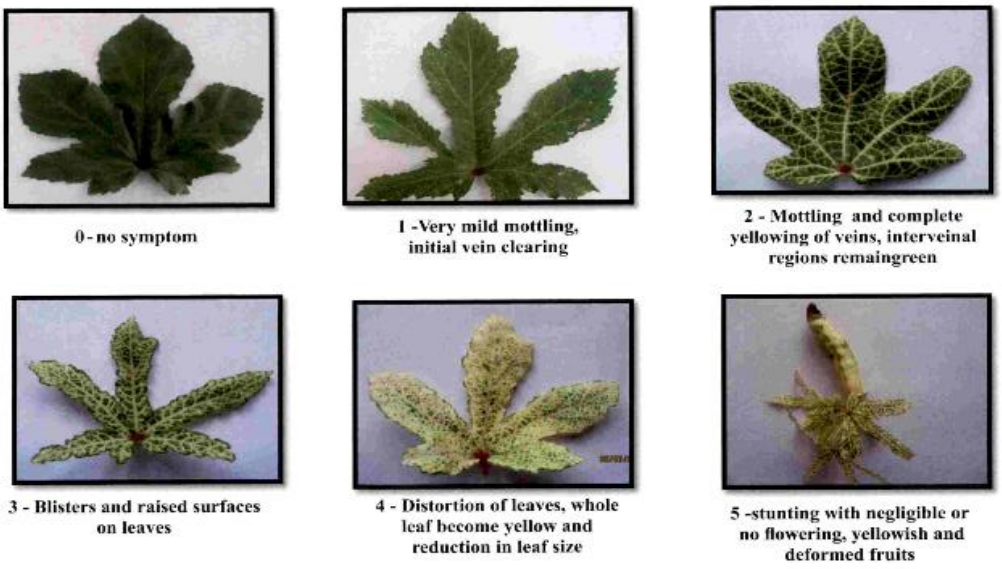

Plate 1. Disease score chart of BYVMD

3.1.2 Disease maintenance by graft inoculation

The virus culture was maintained in susceptible plants by graft inoculation. BYVMV can be transmitted to susceptible rootstock by grafting it with an infected scion of okra. Susceptible root stocks of okra varieties Anakomban and Anjitha was grown in pots. Infected scions (10-15 cm) collected from field were wedge grafted on to susceptible root stock of Anjitha and Anakomban varieties (45 days old stem having pencil thickness). Leaves of root stock and scion (except the apical leaves were removed). Scion base was trimmed to a wedge shape. Root stock was cut at a height of 30 cm from ground and a vertical cleft of same length as wedge (approximately 2 cm) was made. Scion was inserted into cleft on rootstock and wrapped tightly with a cling film. Plant was supported with a twig and covered with a plastic cover containing sufficient small air holes. Grafted plants were kept inside insect-proof glass house.

Symptoms developed on emerging new leaves on root stock below the graft union were observed. The symptoms and the days taken for symptom development were recorded.

3.2 MOLECULAR AND SEROLOGICAL DETECTION

Molecular and serological techniques are important for detection and identification of plant viruses. Molecular detection can be done using virus specific primer and serological detection by using virus specific antibody.

3.2.1. Molecular detection

Molecular detection of plant viruses involves major steps like isolation of viral nucleic acid, agarose gel electrophoresis, quantification using spectrophotometer and PCR

3.2.1.1 Isolation of DNA

DNA virus, BYVMV can be isolated from infected sample by following total genomic DNA extraction protocols like CTAB method (Delleporta *et al.,* 1983).

The liquid nitrogen helps in DNA isolation by enabling the easy rupture of cell wall and by preventing the denaturation of nucleic acid by making cell brittle and inactivating enzymes respectively. The C-TAB buffer (Appendix I) disrupts the cell wall by using a cationic detergent C-TAB (Cetyl Trimethyl Ammonium Bromide). Ethylene Diamine Tetra Acetic Acid (EDTA) inactivates DNase and Tris/HCl provides pH buffering capacity. β- mercaptoethanol denatures protein whereas polyvinyl pyrrolidone (PVP) removes the polyphenolics. The nucleic acid can be separated from proteins and other contaminants using phenol: chloroform: isoamyl alcohol mix. Chilled isopropanol aids the precipitation and separation of DNA.

1. 150 mg of okra leaf tissue was chopped and ground into fine powder with liquid nitrogen using a sterile mortar and pestle.
2. 10 ml preheated CTAB buffer containing 2% β-mercapto ethanol and 2% polyvinylpyrrolidone was poured into powder and mixed well.
3. 1 ml of the homogenate was transferred into 1.5 ml sterile micro centrifuge tube and vortexed for 5-10 seconds.
4. Incubated the mixture at 60ºC for 30 minutes in a heating block and mixed the homogenate by inverting the tubes in every 10 minutes interval.
5. Centrifuged the homogenate at 13000 rpm for 10 minutes at room temperature (Centrifuge 5804 R, Eppendorf, Germany).
6. Supernatant was transferred into a micro centrifuge tube and equal volume of phenol: chloroform: isoamyl alcohol mix (24:1) was added. Mixed the tubes by inversion and centrifuged at 10000 rpm for 5 minutes.
7. The top aqueous layer was transferred into a micro centrifuge tube and centrifuged at 13000 rpm for 10 minutes after adding equal volume of chloroform: isoamyl alcohol mix into it.
8. Transferred the top layer into a new tube using a micro pipette and equal volume of pre chilled isopropanol was added and mixed gently. Incubated it overnight at -20ºC.

9. Centrifuged at 13000 rpm at 4°C for 15 minutes and supernatant was discarded without disturbing the pellet.

10. 5µl 3 M sodium acetate and 100µl 70 % chilled ethanol was added into micro centrifuge tube containing pellet and centrifuged at 10000 rpm at 4°C for 5 minutes.

11. Decant the supernatant and washed the pellet again using 100µl 70 % chilled ethanol by centrifuging at 10000 rpm at 4°C for 5 minutes.

12. Decant the supernatant carefully and air-dried the pellet at room temperature.

13. DNA pellet was dissolved in 30 µl TE buffer (Appendix II) and stored at -20ºC.

3.2.1.2 Agarose gel electrophoresis (AGE)

Separation, identification and purification of DNA fragments can be done using Gel electrophoresis. When an electric field is applied, negatively charged DNA will move towards the anode in an agarose gel matrix. DNA molecules separated based of molecular weight will bind to the ethidium bromide dye in agarose gel matrix and can be visualised using molecular imager Gel Doc^{TM} XR+ with Image Lab software. Presence of DNA is confirmed by running the isolated sample in 0.8 per cent agarose gel.

1. 0.8 g molecular grade agarose powder was dissolved in 100 ml 1X TAE buffer (Appendix III) by melting.
2. 4 µl ethidium bromide was added to it after cooling
3. Cooled agarose was casted in a gel casting tray with a suitable comb.
4. Comb from solidified agarose was carefully removed and transferred it into a horizontal type gel electrophoresis unit (Bio-Rad, USA) filled with 1X TAE buffer.
5. 5µl DNA and 2 µl 6X loading dye were mixed and loaded into wells of agarose gel. 1 kb ladder (GeNei) was also loaded.

6. Run it with power pack unit (Bio-Rad, USA) at 70 V cm^{-1} until optimum separation take place (when dye run three fourth of the total distance)
7. The gel was visualized using a UV trans-illuminator system (Bio-Rad, USA) and documented using gel documentation unit (Gel Doc^{TM} XR+) with an Image Lab software.

3.2.1.3 Quantification of DNA using nanodrop spectrophotometer

The NanoDrop spectrophotometer can determine the quality and quantify DNA, RNA, and protein concentrations by analysing small quantity of sample (1-2 µl). Nucleic acids and purified proteins absorb light with an absorbance peak at 260 nm and 280 nm respectively. Principle of a spectrophotometer is Beer–Lambert law which states that the rate of absorption of monochromatic light at the absorption maxima for a solution is proportional to the solute concentration. For quantification of isolated DNA using a nanodrop spectrophotometer, suitable application (DNA, RNA, or protein) was selected. Cleaned the pedestal with a lint-free wipe and 1 µl sample was placed on pedestal after blanking with 1 µl of TE buffer. The ratio of absorbance at 260 nm and 280 nm along with the concentration of DNA in sample will be displayed on the screen of NanoDrop spectrophotometer.

3.2.1.4 Polymerase Chain Reaction (PCR)

Mullis (1987) discovered PCR which is a method used for detection and amplification of targeted sequence. PCR uses a heat labile polymerase, specific primers, Mg^{2+}, buffer and template for amplifying DNA using a thermocycler. PCR conditions were given in Table 1.

Molecular detection of virus was done using Deng primer (Table 2), universal primer for begomoviruses (Deng *et al*., 1994). A 20 µl reaction mixture using master mix, forward and reverse primers, DNA template (concentration – 150 ng) and nuclease free water was made and amplified using a thermocycler. The components of reaction mixture are shown in Table 3.

Table 1: PCR Reaction condition

Sl. No	Step of PCR	Temperature(ºC)	Duration	No. of cycles
1	Initial Denaturation	95	3 minutes	
2	Denaturation	95	30 seconds	40
3	Annealing	59	1 minutes	
4	Extension	72	1minutes	
5	Final Extension	72	10 minutes	
6	Hold	4		

Table 2. Universal primer used for amplification

Primer	Sequence (5'-3')	Product size (bp)	Reference
Deng 541-F	TAATATTACCKGWKGVCCSC	520	Deng *et al.*,1994
Deng 540-R	TGGACYTTRCAWGGBCCTTCACA		

Table 3: PCR 20 µl reaction mix

Reagents	Volume
2X PCR master mix	10 µl
Forward primer	1.2 µl (1µM)
Reverse primer	1.2 µl (1µM)
Nuclease free Water	4.6 µl
Template	3 µl

3.2.1.5 Agarose gel electrophoresis of PCR products

Presence of virus was confirmed by running the PCR product in 1.2 per cent agarose gel and documented using gel documentation unit (Gel Doc^{TM} XR+) with an Image Lab software.

1. 1.2 g molecular grade agarose powder was dissolved in 100 ml 1X TAE buffer by melting.
2. 4 µl ethidium bromide was added to it after cooling.
3. Cooled agarose was casted in a gel casting tray with a suitable comb.
4. Comb from solidified agarose was carefully removed and transferred it into a horizontal type gel electrophoresis unit (Bio-Rad, USA) filled with 1X TAE buffer.
5. 100bp DNA marker and 5 µl PCR products was loaded into wells of agarose gel.
6. Run it with power pack unit (Bio-Rad, USA) at 70 V cm^{-1} until optimum separation take place (when dye run three fourth of the total distance)
7. The gel was visualized using a UV trans-illuminator system (Bio-Rad, USA) and documented using gel documentation unit.

3.2.1.6 Gel elution of amplicon

The PCR amplicon band at 500 bp was eluted from agarose gel using Geneaid Gel/PCR DNA Fragments extraction kit.

The PCR amplicon band obtained at 520 bp from infected sample was eluted from agarose gel using Geneaid Gel/PCR DNA Fragments extraction kit.

1. Cut out the gel slice containing relevant DNA fragment using a sharp sterile blade and transfer (up to 300 mg) to 1.5 ml micro centrifuge tube
2. Add 500 µl DF buffer to it and vortex
3. Incubate it at 55-60 ºC for 10-15 minutes by inverting the tube in between (ensure gel is completely dissolved)
4. Cool it into room temperature

5. For DNA binding, transfer dissolved sample mixture (≤ 800µl) into a DF column placed in a collection tube (2 ml)
6. Centrifuge it at 16000g for 30 seconds
7. Discard the flow through from collection tube and keep it back
8. Add 600µl washing buffer containing ethanol into DF column and incubate for 1 minute
9. Centrifuge it at 16000g for 30 seconds
10. Discard the flow through from collection tube and keep it back
11. Repeat washing and discard flow through
12. For drying column matrix, centrifuge it at 16000g for 3 minutes
13. For DNA elution, place the dried column matrix into a 1.5 ml microcentrifuge tube
14. Add 30µl ((20-50µl) elution buffer or TE buffer into centre of column matrix
15. Incubate it at least 2 minutes to ensure complete absorption of elution buffer
16. Centrifuge at 16000g for 2 minutes
17. Purified DNA will be obtained in micro centrifuge tube

3.2.1.7 Sequencing of PCR product

Gel eluted PCR product was sequenced at GeneSpec PVT. LTD, Kochi using Sanger sequencing technique.The molecular characterization of the sequenced sample was done using Basic Local Alignment Search Tool (BLAST) a sequence similarity search program in National Centre for Biotechnology Information (NCBI) database. Compared the sample nucleotide sequences to sequence in database and calculated the statistical significance of the matches.

3.2.2 Serological detection

Diseased and healthy samples were diagnosed using enzyme linked immunosorbent assay (ELISA) and dot immunobinding assay (DIBA).

3.2.2.1 Double Antibody Sandwich -Enzyme Linked Immunosorbent Assay (DAS- ELISA)

ELISA is a serological detection method for fast detection of viruses. Capture and detection antibodies are used in Double Antibody Sandwich - ELISA. The assay does not require a purified antigen, but it does require a reporter-labeled detection antibody. In most cases, an antibody labeled with alkaline phosphatase is used. Buffers for DAS- ELISA is listed in Appendix IV.

Polyclonal antisera ToLCNDV- Tomato leaf curl New Delhi virus (DSMZ, Germany) was used for detection. Protocol of DAS- ELISA is as follows.

1) Antibody AS-1109, IgG was diluted in carbonate coating buffer in 1:1000 ratio (1.5µl antibody in 1500µl buffer).
2) 200µl of diluted antibody was loaded into each well of the ELISA microtiter plate.
3) Covered plate was incubated at 37ºC for 2-4 hours.
4) Washed the plate thrice with PBS- Tween using ImmunoWash^{TM} 1575 Microplate Washer.
5) Samples were extracted using sample extraction buffer c 1:10 ratio.
6) 200 µl aliquots of test samples were added into each well and incubated overnight at 4ºC after covering.
7) Washed the plates thrice with PBS- Tween using microplate washer.
8) Secondary antibody anti- rabbit ALP conjugate (AS-1109, IgG-AP) was diluted in PBS-TPO buffer in 1:500 ratio (3µl antibody in 1500µl buffer).
9) 200µl of diluted antibody was loaded into each well and incubated the covered plate at 37ºC for 2 hours.
10) Washed the plates thrice with PBS- Tween using microplate washer.
11) Dissolved substrate p- nitro phenyl phosphate (PNPP) in substrate buffer in 1:1 ratio (5 mg in 5 ml).
12) Added 200 µl of freshly prepared substrate to each well and incubated the covered plate in dark at room temperature for 30 – 60 minutes.

13) Absorbance at 405 nm was measured using BIORAD iMark^{TM} microplate reader 30 minutes and one hour after incubation.

3.2.2.2 Dot Immuno-Binding Assay (DIBA)

When detecting a small amount of antigen, Dot Immunobinding Assay (DIBA) is superior to ELISA. In comparison to polystyrene plates, the nitrocellulose membrane NCM is more sensitive. Since it has a greater binding capacity, even 1ng/ml of antigen can be detected in DIBA. Polyclonal antisera ToLCNDV-*Tomato leaf curl New Delhi virus* (DSMZ, Germany) was used for detection. Buffers used for DIBA are listed in Appendix V. The steps followed are,

1) Extracted the sample in antigen extraction buffer in 1:10 ratio and centrifuged at 10000 rpm for 15 minutes.
2) The supernatant was mixed with chloroform (2:1) and vortexed.
3) Centrifuged it at 12000 g for 2 minutes.
4) The supernatant was mixed with antigen extraction buffer in the ratio of 1:4 and vortexed.
5) Cut out 1x1 cm sized nitrocellulose membrane (NCM) squares.
6) It was blotted in tris buffer saline and air dried after blotting.
7) 10 µl of the sample was placed in the middle of each square and left to dry for 45 minutes.
8) Then immersed it in blocking solution for 1 hour at room temperature.
9) Washed NCM in TBS thrice for 3 minutes.
10) Incubated it at 4ºC overnight in AS-1109, IgG (primary antibody) diluted in TBS SDM.
11) NCM was rinsed in TBS for 3 min thrice and incubated at room temperature for 1 h in secondary antibody (AS-1109, IgG-AP diluted in TBS SDM) with gentle oscillation.
12) Washed NCM in TBS thrice for 3 minutes.
13) Incubated it in Nitro blue tetrazolium and Bromo chloro indolyl phosphate substrate solution in dark at 37 ºC with gentle oscillation.

14) After colour development, NCM was washed in fixing solution for 10 minutes and air dried.
15) Analyzed it using Molecular imager Gel Doc^{TM} XR+ with Image Lab software.

3.3 SEED TRANSMISSION STUDIES

Seed transmission of bhendi yellow vein mosaic virus was studied by grow out test, molecular detection, and serological detection of virus in seed. Seeds were collected from infected plants (Plate 2).

3.3.1 Molecular detection of virus in seed

Random mature seeds collected from BYVM virus infected plants were examined for virus by PCR using universal primer for geminiviruses. Same procedure and conditions mentioned in BYVMV molecular detection was followed for DNA extraction, gel electrophoresis and PCR. Whole seeds were ground for DNA extraction and PCR was done with isolated DNA.

Presence of virus in seed coat, cotyledon and germ tube of random samples of mature seeds collected from BYVM virus infected plants were also checked. Collected seeds were washed thoroughly in running water and then with distilled water. Surface sterilised the seeds by dipping seeds in 0.1 per cent mercuric chloride for 1 minute. Washed the seeds thoroughly in sterile water thrice. Autoclaved tissue paper in petri plate was moistened with sterile water. Seeds were kept on it for germination. After germination, using a sterile blade seed coat, cotyledon and germination tube were separated and molecular detection of virus was done with mentioned protocol (Plate 3)

3.3.2 Grow out test in insect proof glasshouse

Seeds of BYVMV infected okra varieties Anjitha, Anakomban and Arka Anamika were collected from Vellayani, Trivandrum. 286 seeds were sown in pots and germinated plants were maintained in insect proof glass house condition. The symptoms in the emerging seedlings were observed till fruiting. Random seedlings

from the grow out test were examined for presence of virus by PCR using universal primer for geminiviruses (Deng *et al*., 1994).

3.3.3 Grow out test in *in vitro*

Random mature seeds collected from BYVM virus infected plants were placed in half MS media after surface sterilisation. Symptoms in emerging seedling was observed. DNA from leaves of germinated seed was isolated and molecular detection of virus was done using universal primer.

3.3.4 Serological detection of virus in seed and leaves

Serological detection of the presence of virus in grown out plant leaves and random mature seeds collected from BYVM virus infected plants were done using enzyme linked immunosorbent assay (ELISA) as mentioned protocol using polyclonal antisera ToLCNDV- Tomato leaf curl New Delhi virus (DSMZ, Germany).

3.4 SCREENING OF DIFFERENT GENOTYPES OF OKRA BY GRAFT TRANSMISSION

Seeds of different genotypes of okra were collected from National Bureau of Plant Genetic Resources (10) and research station (5) and screened for okra yellow vein mosaic disease. Okra cultivar, Anakomban was used as a susceptible check. Pot culture experiment with 10 treatments and 3 replications was done. Virus infected scions were collected from Anjitha and Anakomban varieties. Wedge grafting was done (Plate 4). The symptoms developed on rootstocks of different genotypes of okra were observed and disease severity based on the vulnerability index was calculated and varieties were categorized into various resistance grades based on the chart. Pot culture experiment.

Design : CRD

Treatments : 15

Replication : 3

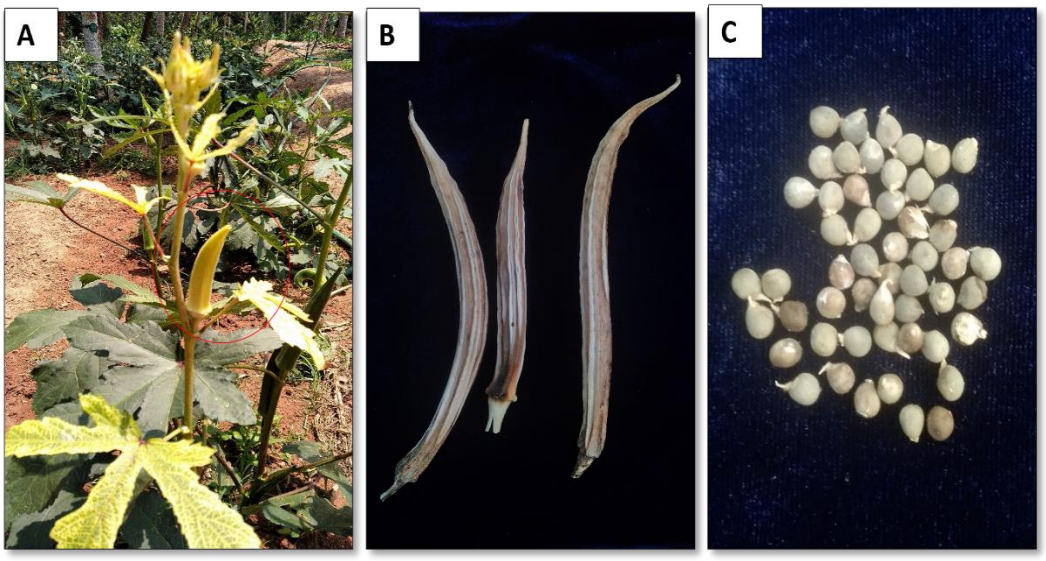

Plate 2. Collection of seeds from BYVMD infected plants. A- Infected plant pod; B- Dried pods; C- Collected seeds

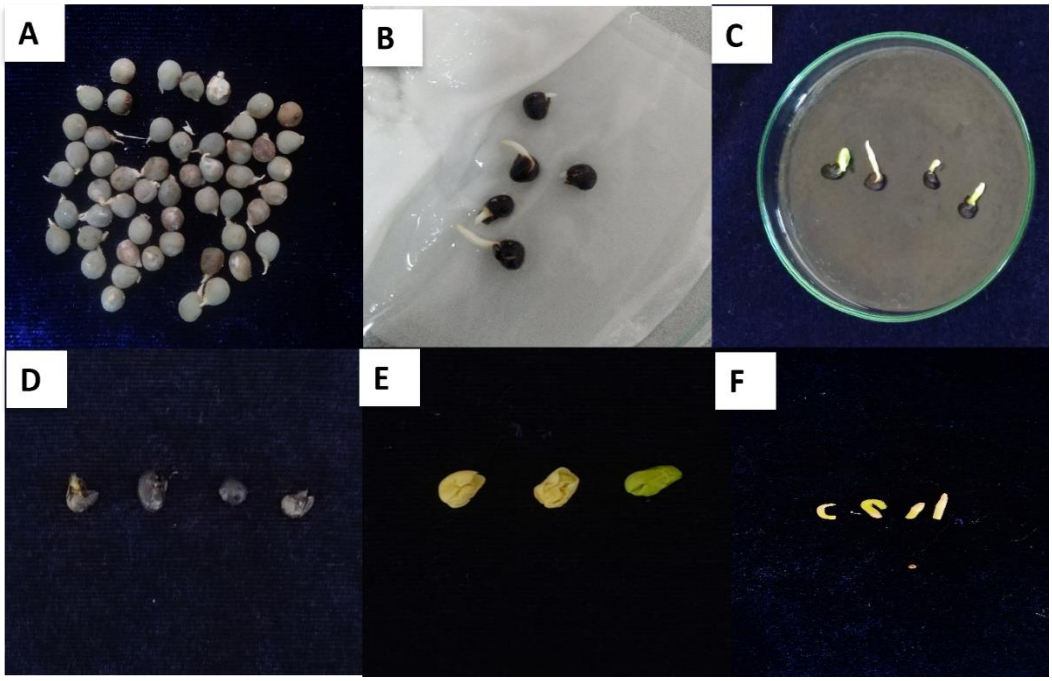

Plate 3. Molecular detection of virus in seed. A- seed collected from infected plants; B, C- Germinated seeds; D- Seed coat; E- Cotyledon; F- Germ tube

Table 4. Okra accessions/ varieties collected for screening of disease resistance

Sl. No.	Treatments	Variety/ Accession no.
1.	T1	IC 052303
2.	T2	IC 00780
3.	T3	IC 588166
4.	T4	IC 002134
5.	T5	IC 006101
6.	T6	IC 002024
7.	T7	IC 043279
8.	T8	IC 093771
9.	T9	IC 093688
10.	T10	IC 045820
11.	T11	Anakomban
12.	T12	Arka Anamika
13.	T13	Anjitha
14.	T14	Phule Vimukta
15.	T15	Arka Nikitha

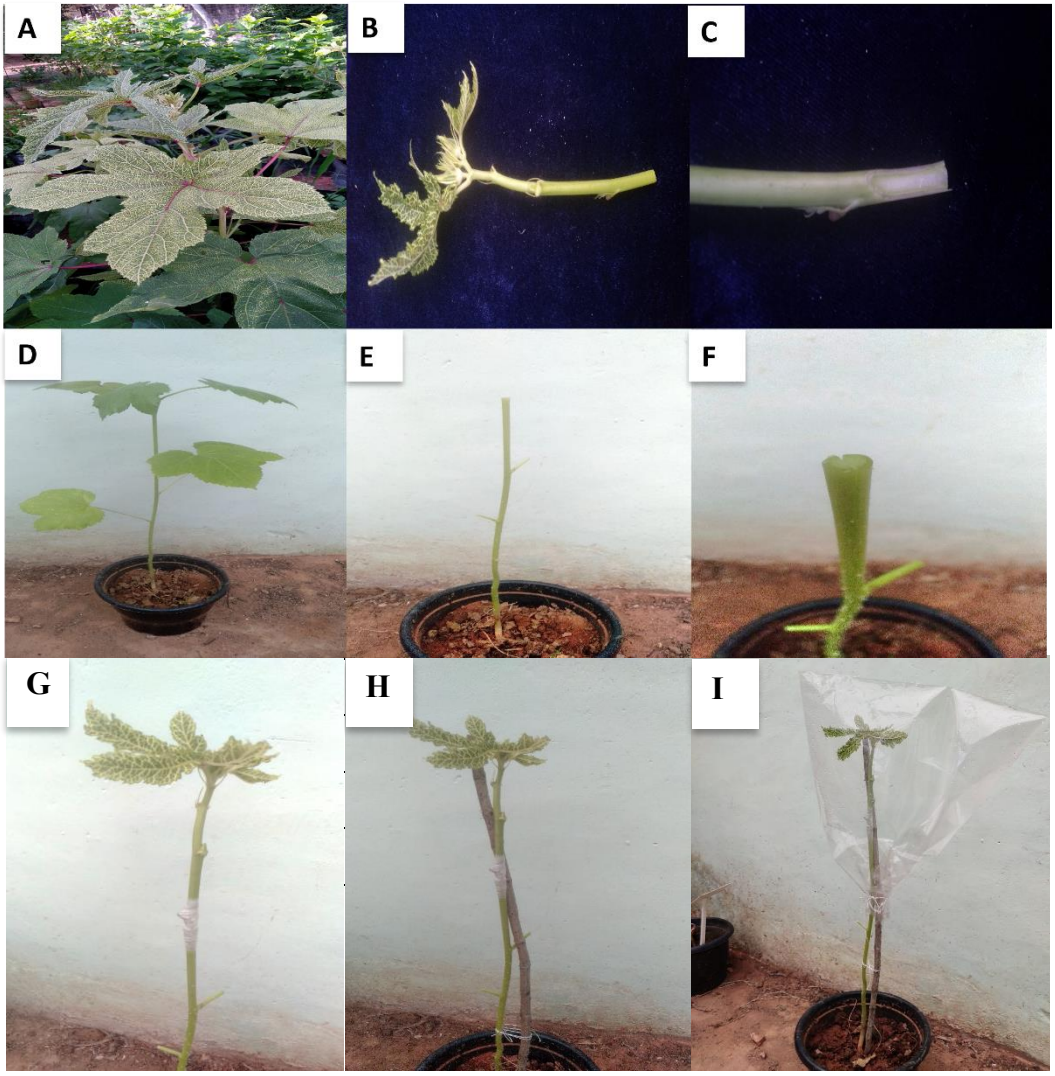

Plate 4. Screening for BYVMD resistance using graft inoculation. A- BYVMD infected plant; B- Infected scion; C- Wedge shaped cut on scion; D- Root stock; E- rootstock without leaves; F- Vertical cut on root stock.

Table 5. Different grades of resistance and susceptibility based on vulnerability index

Grade	Vulnerability index	Reaction category
I	0	Resistant (R)
II	1-25	Moderately resistant (MR)
III	26-40	Moderately susceptible (MS)
IV	41-50	Susceptible (S)
V	>50	Highly susceptible (HS)

3.5 BIOCHEMICAL ANALYSIS

Changes in defence related enzymes such as peroxidase (Srivastava, 1987), polyphenol oxidase (Mayer *et al.,* 1965) and phenylalanine ammonia lyase (Dickerson *et al.,* 1984) was analysed in BYVMV susceptible variety Anakomban and tolerant variety Phule Vimukta. Enzyme activity was recorded as change in absorbance per minute per gram of leaf tissue. Enzyme activity was estimated at 30, 45 and 60 days after graft inoculation. Grafting was done 45 days after sowing.. The enzyme activity at different crop growth stages were also estimated.

Design : CRD

Treatments : 4

Replication : 3

T1: Susceptible variety without graft inoculation

T2: Tolerant variety without graft inoculation

T3: Susceptible variety after graft inoculation

T4: Tolerant variety after graft inoculation

Biochemical analysis of five varieties Anakomban, Arka Anamika, Anjitha, Arka Nikitha and Phule Vimukta was done in different growth stages. PO, PPO and PAL activity in 30,45,75,90 and 105 days after sowing was recorded. PO, PPO and PAL activity of healthy plants and graft inoculated plants of Anakomban, Arka Anamika, Anjitha, Arka Nikitha and Phule Vimukta on 30 days after graft inoculation (75 DAS), 45 days after graft inoculation (90 DAS) and 60 days after graft inoculation (105 DAS) was done. 0.1 M sodium phosphate buffer was used for PO and PPO whereas 0.1 M sodium borate buffer for PAL (Appendix VI).

3.5.1 Peroxidase (PO) assay (Srivastava, 1987)

Srivastava (1987) described the method for estimation of Peroxidase (PO) present in sample. Fresh okra leaf sample was homogenised with 0.1 M sodium phosphate buffer (pH 6.5) and a pinch of poly vinyl pyrrolidone (PVP) using a pre-chilled pestle and mortar. Transferred the homogenate into a micro centrifuge tube. Centrifuged it at 10000 rpm at 4°C for 10 minutes. The supernatant contains the enzyme extract. 3 ml of 0.05 M pyrogallol and 50 µl of enzyme extract were taken in sample cuvette and reference cuvette. Change in absorbance of sample was measured at 420 nm using a spectrophotometer (Systronics UV-VIS spectrophotometer 118). Blanking was done and added 1ml one per cent hydrogen peroxide for initiating the reaction in sample cuvette. Absorbance at an interval of 30 seconds were noted and continued for 180 seconds. PO activity was expressed as change in the absorbance per minute per gram of sample.

3.5.2 Polyphenol oxidase (PPO) assay (Mayer *et al.*, 1965)

Fresh okra leaf sample was homogenised with 0.1 M sodium phosphate buffer (pH 6.5) and a pinch of poly vinyl pyrrolidone (PVP) using a pre-chilled pestle and mortar. Transferred the homogenate into a micro centrifuge tube.

Centrifuged it at 10000 rpm at 4°C for 10 minutes. The supernatant contains the enzyme extract. 1 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 50 µl of enzyme extract were taken in sample cuvette and reference cuvette.

Change in absorbance of sample was measured at 495 nm using a spectrophotometer. Blanking was done with reference cuvette. Addition of one ml of 0.01 M catechol in sample cuvette will initiate the reaction. Absorbance at an interval of 30 seconds were noted and continued for 180 seconds. PPO activity was expressed as change in the absorbance per minute per gram of sample.

3.5.3 Phenylalanine ammonia lyase (PAL) assay (Dickerson *et al.*, 1984)

1 g of okra leaf samples and 5 ml 0.1 M sodium borate buffer of pH 8.8 were homogenized to fine paste using a prechilled mortar and pestle. Centrifuged the homogenate at 10000 rpm at 4°C for 10 minutes and separated the supernatant which is the enzyme extract. The reaction mixture contained 3 ml of 0.1 M sodium borate buffer, 200 µl enzyme extract and 100 µl 12 millimolar l-phenylalanine. But reference contain 3 ml of 0.1 M sodium borate buffer and 200 µl enzyme extract only. Incubated the reaction mixture and blank in glass veils at 40°C for 30 minutes. Added 200 µl 3N HCl immediately after incubation to stop the reactions. Absorbance at 290 nm was detected spectrophotometrically after blanking with reference. PAL activity was determined spectrophotometrically, by production of trans-cinnamic acid from l-phenylalanine. The enzyme activity was expressed as µg cinnamic acid per gram of leaf tissue per minute.

3.6 MERISTEM CULTURE FOR THE PRODUCTION OF VIRUS FREE PLANTS

Meristems were cultured in suitable media in aseptic conditions for differentiating into whole healthy plant. Meristem culture of okra was standardised using meristems collected from in vitro established okra plants from seeds. Production of virus free plants were done from meristem collected from infected plant shoots and seeds of BYVMV infected plants. Anjitha variety was used.

3.6.1 Meristem culture standardisation

Seeds of BYVMV infected plants were collected. Washed the seeds in running water and then with distilled water. Dipped the seeds in distilled water with 3-4 drops of tween-20 for 30 minutes. Washed the seeds in distilled water thoroughly. Different concentration of mercuric chloride 0.05%, 0.1%, 0.2% and 0.3 % were checked and best concentration was selected for procedure. Surface sterilisation was Followed by washing in autoclaved water thrice for 1 minute. All these operations were done inside the running laminar air flow chamber. Surface sterilized seeds were placed in half MS medium (Appendix VII). Seeds germinate and seedlings were established

Meristems (0.3–0.5 mm) were isolated with help of a dissecting microscope using a sterile hypodermic needle and scalpel from the 1-2 weeks old seedling inside a laminar air flow chamber Regeneration percentage in different media was checked. Isolated meristems were cultured in half M S media, M S media and hormonal media (Nair et al., 1979). Hormonal media (Nair et al., 1979) is MS media supplemented with 0.5µM benzyl aminopurine (BAP) + 0.1 µM Naphthalene acetic acid (NAA) + 0.1µM Gibberellic acid (GA3). 2–3 weeks after incubation, the developed meristems were sub cultured on different hormone supplemented media for selecting the suitable media for multiple shoot initiation. MS media with 5µM BAP + 0.1 µM NAA+ 0.1µM GA3(Nair et al., 1979), MS media with 1.0 mg/l BAP + 0.5 mg/l GA3 (Anisuzzaman *et al.*,2010) and MS media with 1.0 mg/l IBA and 1.0 mg/l BAP + 0.02 mg/l GA3 was checked for multiple shoot development Established shoots were separated using a sterile scalpel and placed in root initiation and proliferation media. Checked three media for root initiation MS media with 0.1 mg/l BAP + 0.02 mg/l GA_3, MS media with 1.0 mg/l IBA (Anisuzzaman *et al.*,2010) and MS media with 1.0 mg/l IBA + 1.0 mg/l BAP + 0.02 mg/l GA_3.

pH of all the media were adjusted to 5.8 followed by autoclaving at 121°C for 20 min (1.06 kg/cm). All cultures were incubated in culture room at 24 ± 4°C under cool white fluorescent lamps (Phillips Ltd.) and the light intensity was

maintained at 28–34 mol/m/s giving a photoperiod of 16hrs. Rooted plants were acclimatized in the potting mixture with 1 % MS supplement spray.

3.6.2 Meristem culture from infected plant's seeds and shoot

Seeds and Shoots were collected BYVMV infected Anjitha variety for meristem culture. Meristems separated from infected plant's seeds were cultured by following standardised protocol in 3.6.1. Shoots of infected plants were grown in aseptic conditions by following steps given below.

1) Using a sterile sharp blade, nodal segments of BYVMV infected plants were collected.
2) Washed shoots thoroughly in running water and then with distilled water.
3) Explants were dipped in 0.1% Tween 20 for 30 minutes.
4) washed thrice in distilled water.
5) Surface sterilized the explants by dipping in 0.1% mercuric chloride for 3 minutes inside the laminar air flow chamber.
6) Removed mercuric chloride by dipping explants in autoclaved sterile water for 3 minutes (thrice by changing water).
7) Trimmed the ends of explants using a sterile blade.
8) Transferred the explants aseptically into sterile half MS media.
9) Meristems taken from shoots were cultured by following the same protocol in 3.6.1.

3.6.3 Virus indexing

Virus indexing was performed using PCR with the universal Deng primer for begomoviruses. PCR was done with universal primer for begomoviruses to check the absence/presence of BYVM virus in meristem cultured plants. BYVMV infected leaf sample was used as positive control.

Results

4. RESULTS

The research entitled 'Biochemical response of okra to *Bhendi Yellow Vein Mosaic Virus* and production of virus free plants' was undertaken at the College of Agriculture, Vellayani, Thiruvananthapuram during the years 2021 to 2023. The results of the experiments are listed in detail in this chapter.

4.1 COLLECTION AND MAINTENANCE OF VIRUS INFECTED PLANTS

Samples were collected from various locations and symptoms were studied. Virus inoculum was maintained by graft inoculation in susceptible varieties Anjitha and Anakomban.

4.1.1 Collection of BYVMV infected samples and symptomatology

Purposive sampling survey was done and samples were collected from Thiruvananthapuram, Thrissur, and Palakkad areas of AEU 8 (Plate 5) and AEU10 (Plate 6) respectively during the year 2022-2023. The symptomatology, per cent disease incidence (PDI) and vulnerability index (VI) was accessed in the surveyed locations. All plots of surveyed locations were severely infected with okra yellow vein mosaic disease.

Symptomatology of BYMD infected okra plants revealed typical symptoms of vein clearing, vein thickening and distortion of the leaves (Plate 7). In advanced stages, fruits were also found affected and bleached appearance of fruits was observed in the disease infected plants. Early infected plants were observed with stunted growth. Most of the plants were observed to exhibit symptoms during the flowering and fruiting stage. Leaf curling, increase in leaf size, and long petiole were observed in some plants. In the okra variety, Anjitha, floral deformity, vein clearing on flower buds along with bleached appearance of fruits was observed. Various symptoms observed in surveyed locations are listed in Table 6.

The disease incidence of virus infected okra plants in surveyed locations ranged from 37 % to 100 %. The vulnerability index of various BYVMD infected plants varied from 23.40 to 85.20 on assessment of disease severity at different locations cultivating different varieties (Table 7). The vulnerability index was calculated based on a 0-5 disease scale prepared based on the disease symptoms observed in the field. Anakomban, Arka Anamika, Varsha Uphar and Anjitha were the varieties found at the surveyed locations. Hundred per cent okra yellow vein mosaic disease incidence was observed in Anakomban and Varsha Uphar variety in AEU 10 and disease incidence was comparatively less in AEU8, where Anjitha was cultivated along with Anakomban and Arka Anamika. Highest V. I. was observed in Arka Anamika (85.2) followed by varieties, Varsha Uphar (80) and Anakomban (83.53). The okra variety, Anjitha, recorded the lowest disease incidence of 37.20 per cent and vulnerability index of 23.40 .

Disease incidence was observed to be high at the flowering and fruiting stage of okra plants. Plants infected at early stages were showing more vulnerability index than plants infected in later stages. Weed species *Ageratum conyzoides* in field with yellow vein mosaic symptoms were also collected along with crop samples.

4.1.2 Disease maintenance by graft inoculation

Virus was maintained by graft inoculation. Infected scions were wedge grafted onto the root stocks of Anjitha and Anakomban varieties of okra. The grafted plants were kept in insect -proof glass house and observed for symptom development. The symptom of vein clearing was observed in newly emerged leaves from the root stock after 8-9 days of graft inoculation (Plate 8). About 87.50 per cent transmission of the virus was observed in the inoculated plants. One hundred per cent graft transmission was shown in the Anakomban variety and 83% transmission was observed in the Anjitha variety (Table 8). Even though graft union was not observed in all plants there was successful transmission of virus by graft inoculation.

Table 6. Per cent disease incidence and symptoms observed in different locations of AEU 8 and AEU 10

Sl. No.	AEU	Location	GPS coordinates	Variety	Symptoms observed	Total no. of plants	No. of infected plants	Disease incidence (%)
1	AEU-8 Southern laterites	Vellayani (Thiruvananthapuram)	8°43'12".0"N 76°98'75".0"E	Anakomban (2locations)	Mottling, vein clearing, vein thickening and bleaching of fruits	65	41	63.07
				Anjitha (3locations)	Vein clearing, vein thickening, slight curling and increase in leaf size along with long petiole. Floral deformity, vein clearing on flower buds and bleached appearance of fruits, Stunted growth	860	320	37.20
				Arka Anamika	Mottling, vein clearing, vein thickening of leaves, stunted growth and bleaching of fruits	110	101	91.81
	TOTAL					1035	462	44.64
2.	AEU-10 North central laterites	Vellanikkara (Thrissur)	10°32'55".0"N 76°16'58".0"E	Varsha Uphar	Mottling, vein clearing, vein thickening and bleaching of fruits	100	100	100
		Kumbidi (Palakkad)	10°83'07".0"N 76°05'06".0"E	Anakomban	Mottling, high vein clearing, vein thickening and bleaching of fruits	20	20	100
TOTAL						120	120	100

Table 7. Disease severity of okra varieties in surveyed locations

Sl. No.	Variety	Crop stage	Symptoms observed	Vulnerability index	Time of observation
1	Anakomban	Flowering and fruiting stage	Mottling, vein clearing, vein thickening and bleaching of fruits	83.53	May 2023 August 2022
2.	Anjitha	Flowering and fruiting stage	Vein clearing, vein thickening, slight curling and increase in leaf size along with long petiole. Floral deformity, vein clearing on flower buds and bleached appearance of fruits, Stunted growth	23.4	May 2023
3.	Arka Anamika	Flowering and fruiting stage	Mottling, vein clearing, vein thickening of leaves, stunted growth and bleaching of fruits	85.2	July 2022
4.	Varsha Uphar	Flowering and fruiting stage	Mottling, vein clearing, vein thickening and bleaching of fruits	80	July 2023

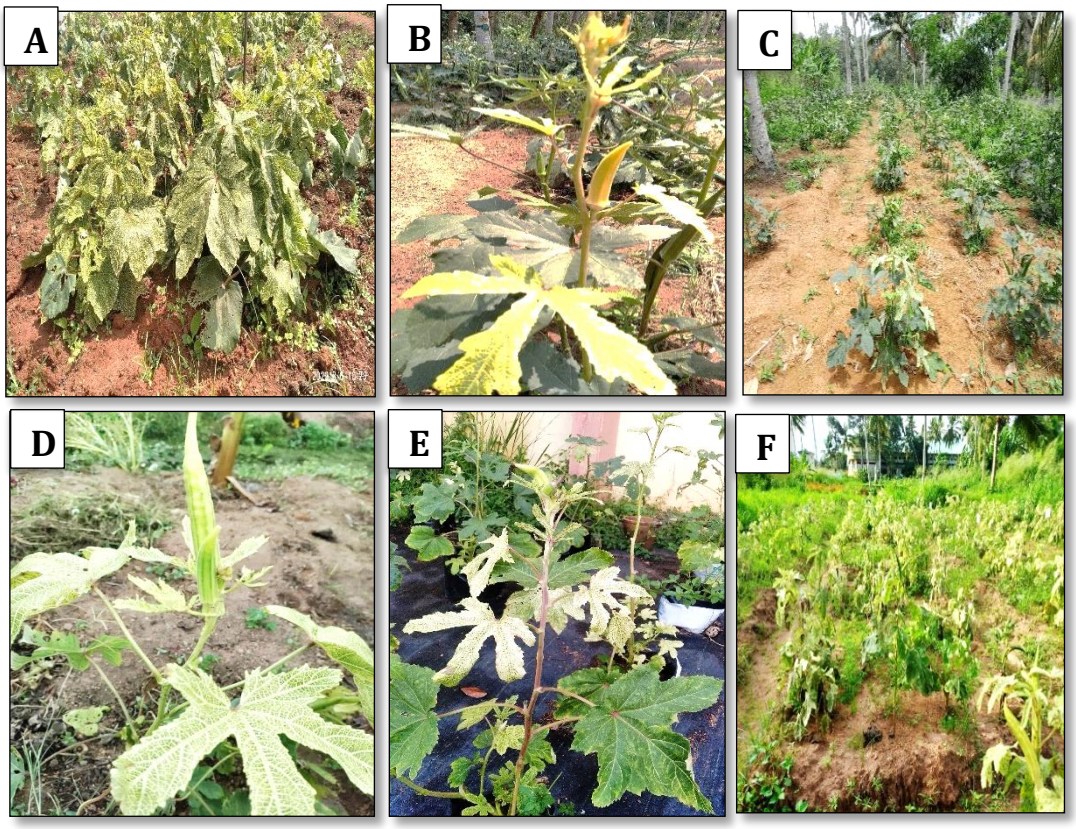

Plate 5. BYVMD symptoms observed in AEU-8 (Vellayani, Thiruvananthapuram). A, B, C- Anjitha: D-Anakomban; E- Arka Anamika

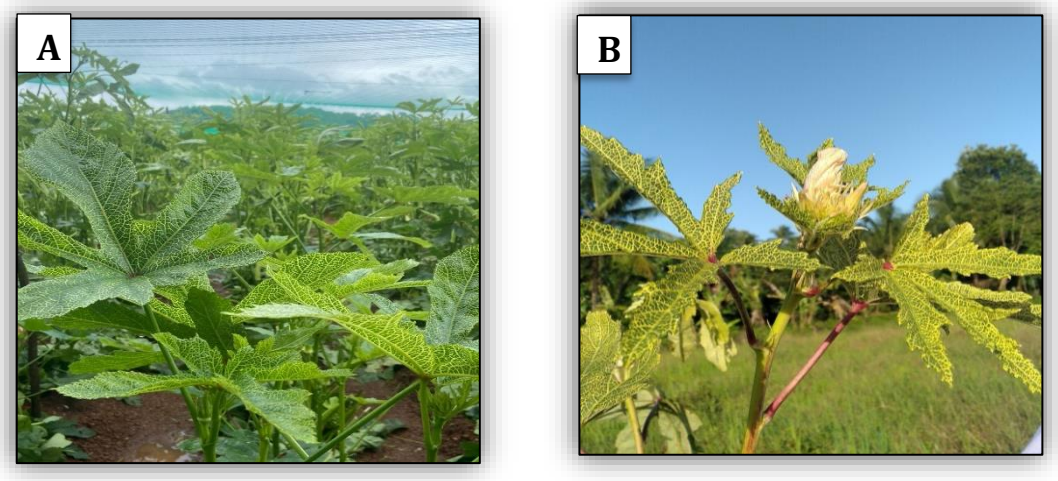

Plate 6. BYVMD symptoms observed in AEU-10. Varsha Uphar (Vellanikkara, Thrissur); Anakomban (Kumbidi, Palakkad)

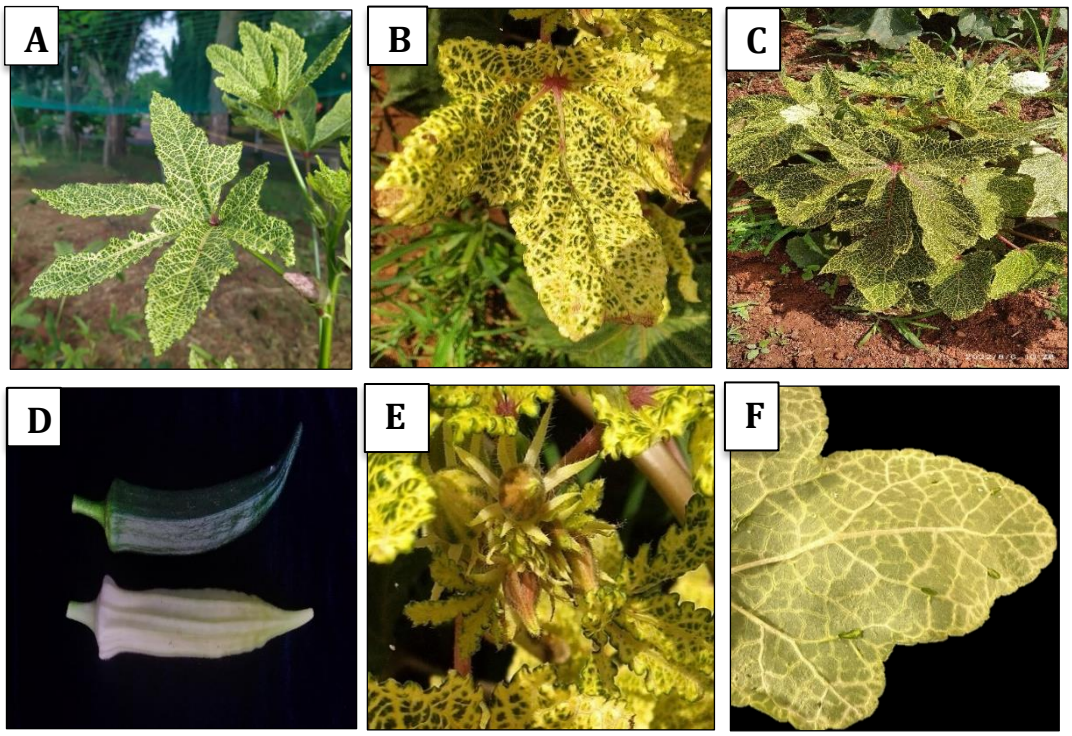

Plate 7. Symptoms observed in BYVMD infected okra plants. A-Vein clearing; B- Crinkling of leaves; C- Stunting of plant; D- bleaching of fruits; E- Floral deformity; F-Enation

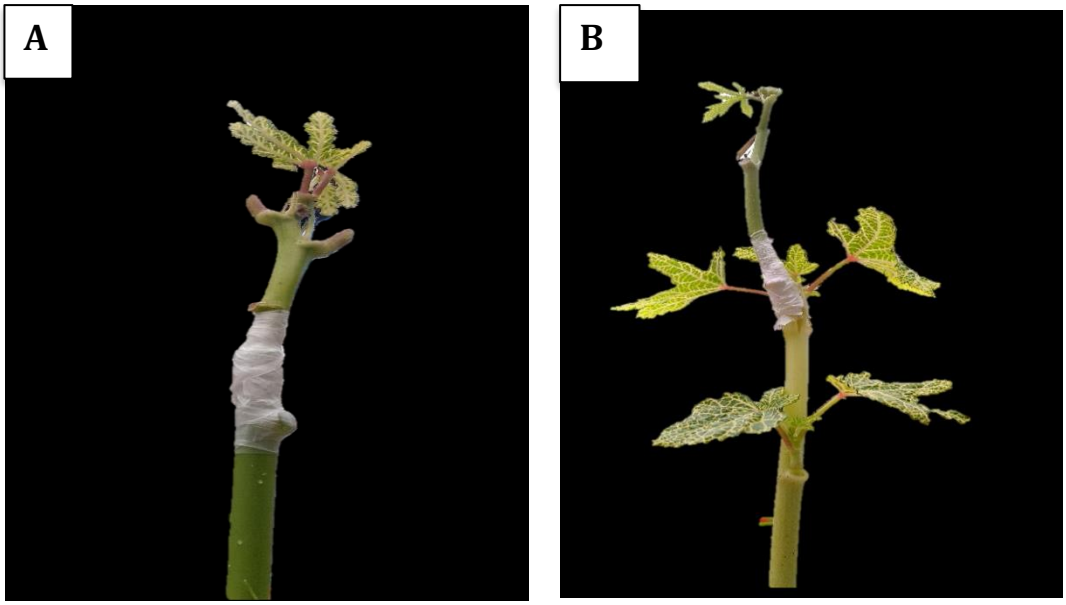

Plate 8. Disease maintenance by graft inoculation. A-Infected scion grafted on susceptible root stock; B- Symptom on newly emerged leaves of rootstock.

Table 8. Disease maintenance by graft inoculation

Sl. No.	Variety	No. of grafted plants	No. of infected plants	Nature/ type of symptoms	No. of days taken for symptom development
1.	Anjitha	30	25	Vein clearing and vein thickening	9
2.	Anakomban	10	10	Vein clearing and vein thickening	8
	Total	40	35		

4.2. MOLECULAR AND SEROLOGICAL DETECTION

Molecular detection was done using universal primer for *Begomovirus* and serological detection was done using tomato leaf curl New Delhi Virus (ToLCNDV) antiserum.

4.2.1. Molecular detection

Isolation of viral nucleic acid, agarose gel electrophoresis, quantification using spectrophotometer and PCR of BYVMV infected samples were done.

4.2.1.1 Isolation of DNA

The total DNA from infected okra leaf samples were isolated using modified CTAB method. DNA was isolated from 150 mg infected okra leaf samples homogenized using 10 ml pre heated CTAB buffer. The white colored pellets were obtained. It was dissolved and diluted in TE buffer to a volume of 30 µl. The total DNA was also isolated from the infected weed plant, *Ageratum conyzoides* with vein clearing symptom collected from BYVMD infected field.

4.2.1.2 Agarose gel electrophoresis

Good quality bands of isolated total DNA were observed in the 1 per cent agarose gel visualized using the gel documentation unit after gel electrophoresis and thereby confirmed the presence of DNA in pellets isolated using modified CTAB method

4.2.1.3 Quantification of DNA using nanodrop spectrophotometer

The quality and quantity of total DNA isolated from infected okra samples were accessed using nanodrop spectrophotometer. Isolated DNA of Arka Anamika (Vellayani) isolate had the highest quantity of 806.5 ngµl^{-1} (Table 9).

Table 9. Quality and quantity of DNA of BYVMV isolates

Sl. No.	Isolate name	OD at 260 nm	OD at 280 nm	260/280 value	Quantity of DNA (ng µl^{-1})
1.	Anakomban, (Vellayani)	4.44	2.09	2.12	146.4
2.	Anjitha (Vellayani)	6.98	3.49	2.00	230.4
3.	Arka Anamika (Vellayani)	24.44	12.60	1.94	806.5
4.	Varsha Uphar (Vellanikkara)	8.91	4.24	2.10	293.9
5.	Anakomban (Kumbidi)	11.04	5.04	2.19	364.5

OD- optical density

4.2.1.4 Polymerase Chain Reaction (PCR)

PCR was done with isolated DNA of BYVMV isolates using universal primer for begomoviruses Deng primer under specified conditions (Table 2). Among different annealing temperatures tried, 59º C showed the best band in gel electrophoresis. Thus, confirmed the primer annealing temperature as 59ºC.

4.2.1.5 Agarose gel electrophoresis of PCR products

All PCR products of BYVMV isolates yielded an amplicon size of 520 bp with Deng primer (Plate 9) and thus confirmed the presence of BYVMV Table 10.

Table 10. Molecular detection of BYVMD isolates using Deng primer

Sl. No.	Location	Variety	Status of Amplicon (+/-)
1.	Vellayani, Thiruvananthapuram	Anakomban	+
		Anjitha	+
		Arka Anamika	+
2.	Kumbidi, Palakkad	Anakomban	+
3.	Vellanikkara, Thrissur	Varsha Uphar	+

3.2.1.6 Gel elution of amplicon

The amplicon obtained from PCR of DNA extracted from okra leaf with vein clearing symptom, enation symptom and *Ageratum conyzoides* with vein clearing were eluted (Plate 10).

3.2.1.7 Sequencing of PCR product

The amplicons obtained in PCR were further sequenced for the molecular characterization of the virus. Amplicons obtained from DNA isolated from leaf samples showing different symptoms were sequenced. The BLAST analysis of the sequence of amplicon from okra with vein clearing symptom showed 96.27 per cent similarity with DNA-A segment of bhendi yellow vein mosaic virus isolate. BLAST analysis result is given in Table 11. The sequence of amplicon from okra sample with enation symptom also showed 96.67 per cent homology with DNA-A segment of bhendi yellow vein mosaic virus (Table 12). The sequence of amplicon from *Ageratum conyzoides* with vein clearing symptom showed 93.99 per cent homology with coat protein of french bean leaf curl Madkeri virus isolate and 89.46 per cent homology with DNA-A segment of ageratum yellow vein Sri lanka virus (Table 13).

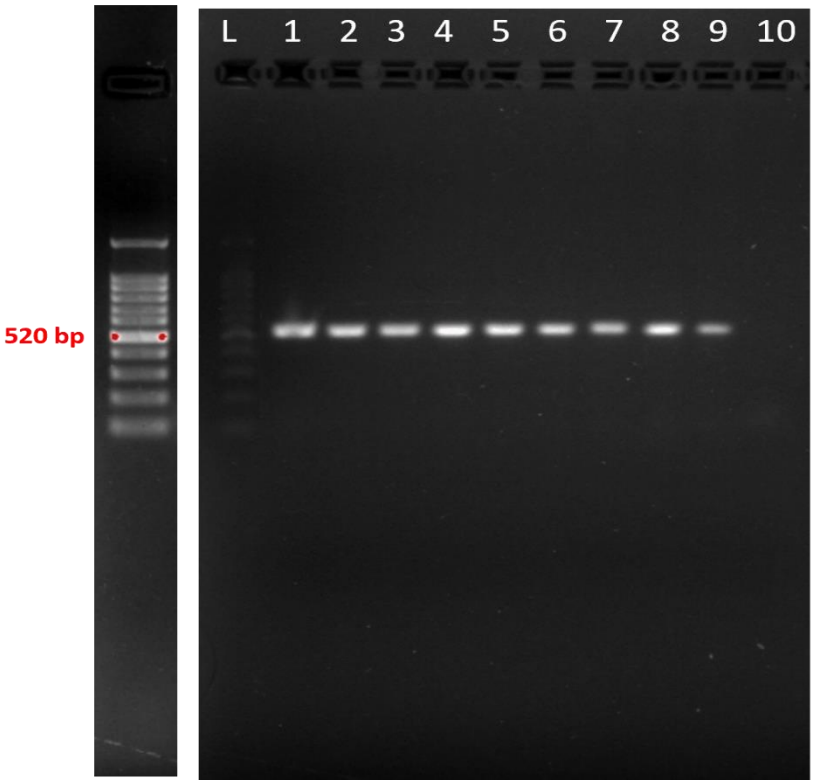

Plate 9. 1.2 % agarose gel profile of DNA samples amplified using DENG primer. lane L- 100 bp DNA marker; 1,2- Anakomban (Vellayani); 3,4- Anjitha (Vellayani); 5,6- Arka Anamika (Vellayani); 7,8- Anakomban(Palakkad); 9- Varsha Uphar (Vellanikkara); 10- Negative control

Plate 10. 1.2 % agarose gel profile of DNA samples amplified using DENG primer. lane L- 100 bp DNA marker; A. Sample with vein clearing symptom; B. Sample with enation symptom

Sequence obtained from okra leaf sample with vein clearing symptom amplified using Deng primer

GGGTCCATAACTCGCGCGATCTTTTTCCAGAACTCGCGCGAGTGGTGG
GTCCAGAACGCAGGACTATGCAGACTCAAAGCTTAGATAACGCTCCTT
CGGCTATAAGTAGTGCGCACTAAGTTTCAATTCAAAAAATGTGGGATC
CACTATTAAACGAATTTCCGGATACGGTTCACGGGTTTCGTTGTATGCT
ATCTGTAAAATATTTGCAACTTTTGTCGCAGGATTATTCTCCAGATACG
CTTGGGTACGAGTTAATACGGGATTTAATTTGTTTTTACGCTCCCGTAA
TTATGTCGAAGCGTCCTGCCGATATCGTCATTTCTACGCCCGCGTCGAA
AGTACGCCGGCGTCTGAACTTCGGCAGCCCATACACCAGCCGTGCTGC
TGCCCCCACTGTCCGCGTCACAAGATCACGAATGTGGGCCAACAGGCC
TATGAACAGGAAACCCAGAATGTACCGGATGTACAGAAGTCCGGATG
TTCCAAGGGGATGTGAAGGCCCATGCAAAGTCC

Sequence obtained from okra leaf sample with enation symptom amplified using Deng primer

TAATATTACCGGTGGGCCGCGCGATTTTTTTAAGTGGTGGGTCCAGAA
CTCGCGCGATCTTTTTCCAGAACTCGCGCGAGTGGTGGGTCCAGAACG
CACGACTATGCAGACTCAAAGCTTAGATAACGCTCCTTCGGCTATAAG
TACGTGCGCACTAAGTTTCAATTCAAAAAATGTGGGATCCACTATTAA
ACGAATTTCCGGATACGGTTCACGGGTTTCGTTGTATGCTATCTGTAAA
ATATTTGCAACTTTTGTCGCAGGATTATTCTCCAGATACGCTTGGGTAC
GAGTTAATACGGGATTTAATTTGTATTTTACGCTCCCGTAATTATGTCG
AAGCGTCCTGCCGATATCGTCATTTCTACGCCCGCGTCGAAAGTACGC
CGGCGTCTGAACTTCGGCAGCCCATACACCAGCCGTGCTGCTGCCCCC
ACTGTCCGCGTCACAAAATCACGAATGTGGGCCAACAGGCCTATGAAC
AGGAAACCCAGAATGTACCGGATGTACAGAAGTCCGGATGTTCCAAG
GGGATGTGAAGGCCCATGCAAAGTCCAA

Sequence obtained from *Ageratum conyzoides* leaf sample with vein clearing symptom amplified using Deng primer

CACCAAGTAGTGGGATTTGTCATCATGTGGGATCCTCTTTTACACGAGT
TTCCCGACACTGTTCACGGTTTTCGGTGTATGCTTGCTGTGAAATACTT
ACAGTTAGTTGGGGATACTTATTCGCCTGATACTTTGGGTTACGATTTA
ATACGTGACCTAATCGCAGTCATTAGAGCAAAGAATTATGTCGAAGCG
TCCAGCAGATATAATCATTTCCACGCCCGCATCAAAGGTGCGTCGCCG
TCTGAACTTCGAGAGCCCGTACACGGGTCGTGCTGCTGTCCCCACTGT
CCGCGTCACAAAGTCAAGAGCATGGGCCAACAGGCCCATGA

Table 11: BLAST analysis of the sequence of amplicon obtained from PCR using Deng primer (vein clearing symptom on okra leaves)

Sl. No.	Similarity to other viruses	Query value	E Value	Percentage similarity (%)	Accession number
1.	Okra leaf curl virus-[India: mau:2012] isolate Mau2 segment DNA-A	97%	0.0	96.27	KF515621.1
2.	Bhendi yellow vein mosaic virus isolate WB1 segment DNA-A	97%	0.0	96.27	KJ462082.1
3.	Bhendi yellow vein mosaic virus [India: Sonipat: OY83:2005] segment DNA-A	97%	0.0	96.27	GU112075.1
4.	Bhendi yellow vein mosaic virus [India: Raichur: OY56:2005] segment DNA-A	97%	0.0	96.27	GU112074.1
5.	Bhendi yellow vein mosaic virus [India: Palaghat:OY138a:2006] segment DNA-A	97%	0.0	96.27	GU112072.1
6.	Bhendi yellow vein mosaic virus segment a clone 10c-RCA-AII-F	97%	0.0	96.09	FN645923.1
7.	Bhendi yellow vein mosaic virus [India: Pandarahalli:OY173:2006] segment DNA-A	97%	0.0	96.08	GU112073.1
8.	Bhendi yellow vein mosaic virus [India: Tirupathi:OY98:2005] segment DNA-A	97%	0.0	96.08	GU112009.1
9.	Bhendi yellow vein mosaic virus clone viro 505 segment DNA-A	97%	0.0	95.88	KF471063.1
10.	Bhendi yellow vein mosaic virus [India: Raichur: OY56:2005] segment DNA-A	97%	0.0	95.69	GU112070.1

Table 12: BLAST analysis of the sequence of amplicon obtained from PCR using Deng primer (enation symptom on okra leaves)

Sl. No.	Similarity to other viruses	Query value	E Value	Percentage similarity	Accession number
1.	Okra leaf curl virus [India: Mau:2012] isolate Mau2 segment DNA-A	96%	0.0	96.67	KF515621.1
2.	Bhendi yellow vein mosaic virus isolate WB1 segment DNA-A	96%	0.0	96.67	KJ462082.1
3.	Bhendi yellow vein mosaic virus [India: Sonipat: OY83:2005] segment DNA-A	96%	0.0	96.67	GU112075.1
4.	Bhendi yellow vein mosaic virus [India: Raichur: OY56:2005] segment DNA-A	96%	0.0	96.67	GU112074.1
5.	Bhendi yellow vein mosaic virus segment A clone 10c-RCA-AII-F	96%	0.0	96.49	FN645923.1
6.	Bhendi yellow vein mosaic virus [India: Pandarahalli:OY173:2006] segment DNA-A	96%	0.0	96.49	GU112073.1
7.	Bhendi yellow vein mosaic virus [India: Palaghat:OY138A:2006] segment DNA-A	96%	0.0	96.49	GU112072.1
8.	Bhendi yellow vein mosaic virus [India: Tirupathi:OY98:2005] segment DNA-A	96%	0.0	96.49	GU112009.1
9.	Bhendi yellow vein mosaic virus clone VIRO 505 segment DNA-A	96%	0.0	95.30	KF471063.1
10.	Bhendi yellow vein mosaic virus [India: Raichur: OY56:2005] segment DNA-A	96%	0.0	95.12	GU112070.1

Table 13. BLAST analysis of the sequence of amplicon obtained from PCR using Deng primer (vein clearing symptom on *Ageratum* leaves)

Sl. No.	Similarity to other viruses	Query value	E Value	Percentage similarity	Accession number
1.	French Bean leaf curl Madkeri virus isolate FB1 pre-coat protein (AV2), coat protein(AV1)	96%	1e-137	93.99	MK087122.1
2.	Ageratum yellow vein Sri Lanka virus segment DNA-A	96%	1e-112	89.46	NC 002981.1
3.	Ageratum yellow vein Sri Lanka virus segment DNA-A	96%	1e-112	89.46	AF314144.1
4.	Passion fruit leaf curl Madkeri virus isolate PF1 segment DNA-A pre-coat protein (AV2), coat protein (AV1)	96%	6e-106	88.29	NC 076047.1
5.	Syndrella yellow vein clearing virus isolate NCBS-PS-1 segment A	96%	6e-91	85.59	NC 038994.1
6.	Tomato leaf curl Kerala virus isolate RM429	96%	3e-79	84.74	KY926897.1
7.	Tomato leaf curl Kerala virus isolate RM430	96%	1e-77	84.42	KY926898.1
8.	Tomato leaf curl Kerala virus isolate RM425	96%	1e-77	84.42	KY926894.1
9.	Cotton leaf curl Kokhran virus isolate SR3	96%	1e-77	84.47	MF774564.1
10.	Tomato leaf curl Mumbai virus clone PBAMM9 DNA-A	96%	2e-76	85.03	MH577037.1

4.2.2 Serological detection

Diseased and healthy samples were diagnosed using enzyme linked immunosorbent assay (ELISA) and dot immunobinding assay (DIBA).

4.2.2.1 Double Antibody Sandwich -Enzyme Linked Immunosorbent Assay (DAS- ELISA)

DAS- ELISA was done using Polyclonal antisera ToLCNDV (DSMZ, Germany) for detecting the presence of virus in leaf samples showing vein clearing along with healthy control. Positive reaction was shown by all the infected samples tested.

Table 14: Reaction of BYVMD infected samples in DAS - ELISA

Sl. No.	Sample variety	Antiserum	Absorbance value at 415nm*		Ratio of absorbance at 415nm	Remark
			Diseased	Healthy		
1	Anjitha	ToLCNDV	0.17	0.007	24.29	Positive

* Mean of 3 replications

Leaves of healthy and BYVMD infected okra were assayed using ToLCNDV (DSMZ, Germany) polyclonal antiserum. Healthy control and buffer control along with three diseased samples were analysed using ELISA. Healthy control and buffer control have no reaction in ELISA. BYVMD infected samples showed 24.29 percentage increase over healthy samples. The gel documentation analysis report of BYVMD infected samples in DIBA is shown in Table 14.

4.2.2.2 Dot Immuno-Binding Assay (DIBA)

DIBA was done using Polyclonal antisera ToLCNDV (DSMZ, Germany) for detecting the presence of virus in leaf samples showing vein clearing along with healthy and buffer control Plate 11. Positive reaction was shown by all the infected

samples tested. BYVMD infected samples showed 37.47 % increase over healthy. Healthy and buffer control showed negative values (there was no colour reaction on nitrocellulose membrane) which indicate the absence of virus in it. DIBA report is shown in table 15.

Table 15. Reaction of BYVMD infected samples in DIBA with ToLCNDV antiserum

Sl. No.	Type	Volume intensity	Area (mm^2)	Average Volume	Per cent increase over healthy
1	Diseased	1419.4	34	1138.95	37.47
		948.5	27.4		
2	Healthy	817.1	34	828.5	0
		839.9	27.4		
3	Buffer control	715.9	34	730.15	-
		744.4	27.4		

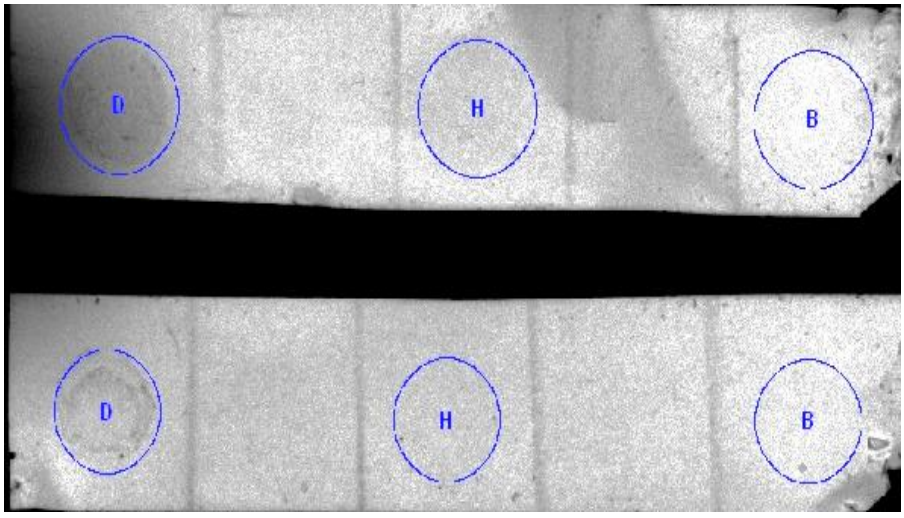

Plate 11. Color development on NCM on DIBA analysis of BYVMD infected samples with ToLCNDV antiserum. D- BYVMD infected sample; H- Healthy control; B- Buffer control

4.3 SEED TRANSMISSION STUDIES

Seed transmission of *Bhendi yellow vein mosaic virus* was studied by grow out test, molecular detection and serological detection of virus in seed. Seeds were collected from okra plants with severe BYVMD infection.

4.3.1 Molecular detection of virus in seed/ seed parts

Presence of virus in whole seed, seed coat, cotyledon and germ tube of random samples of mature seeds collected from BYVM virus infected plants were checked. Presence of virus was detected in seed (Table 16). The percent disease infestation in seed is 20 %. (Plate 12,13).

Table 16. Molecular detection of seed infestation percentage of BYVMD

Sl. No	Type of seed sample	No. of seeds/seed parts accessed	No. of seeds/ seed parts with amplicon	Seed infestation percentage
1	Whole seed	20	4	20
2	Seed coat	10	0	0
3.	Endosperm	10	0	0
4.	Germ tube	10	0	0

4.3.2 Grow out test in insect proof glass house

Out of 286 seeds sown, 154 seeds germinated and none of the plants showed BYVMD symptom (Plate 14). Leaves were asymptomatic till last stage of plants (Plate 15). Therefore, the D.I and V. I. are zero (Table 17). Random seedlings from the grow out test were examined for presence of virus by PCR using universal primer for geminiviruses (Plate 16).

Table 17. Grow out test of seeds collected from BYVMD infected plants

Sl. No.	Variety	No. of seeds sown	No. of seeds germinated	Germination percentage	No. of infected plants	Status of amplicon (+/-)
1	Anjitha	170	88	51.76	0	-
2	Anakomban	16	10	62.5	0	-
3	Arka Anamika	20	0	0	0	-
4.	Varsha Uphar	80	56	70	0	-
	Total	286	154	57.14	0	

4.3.3 Grow out test in *in vitro*

No symptoms of BYVMD were shown by the emerging seedlings of seeds of BYVM virus infected plants placed in half MS media. Germination percentage was less and no seeds of Arka Anamika were germinated. DNA from leaves of germinated seed was isolated and molecular detection of virus was done using universal primer. No amplicon was obtained in PCR analysis showing the absence of virus in plants raised from the seeds from infected plants. Variety and percentage germination of seeds collected from infected plants are shown in table 18.

Table 18. Grow out test in *in vitro*

Sl. No	Sample variety	No. of seeds placed	No. of seeds germinated	Germination percentage	Amplicon (present/ absent)
1	Anjitha	10	3	30	Absent

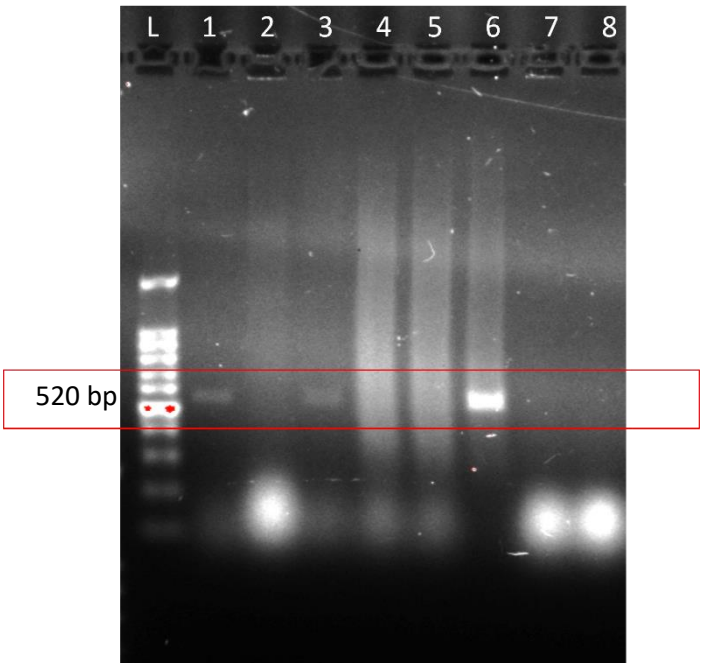

Plate 12. 1.2 % agarose gel profile of DNA from whole seed amplified using DENG primer. lane L- 100 bp DNA marker; 1,3,4,5- Positive seed sample; 6- Positive control; 7,8- Negative seed sample.

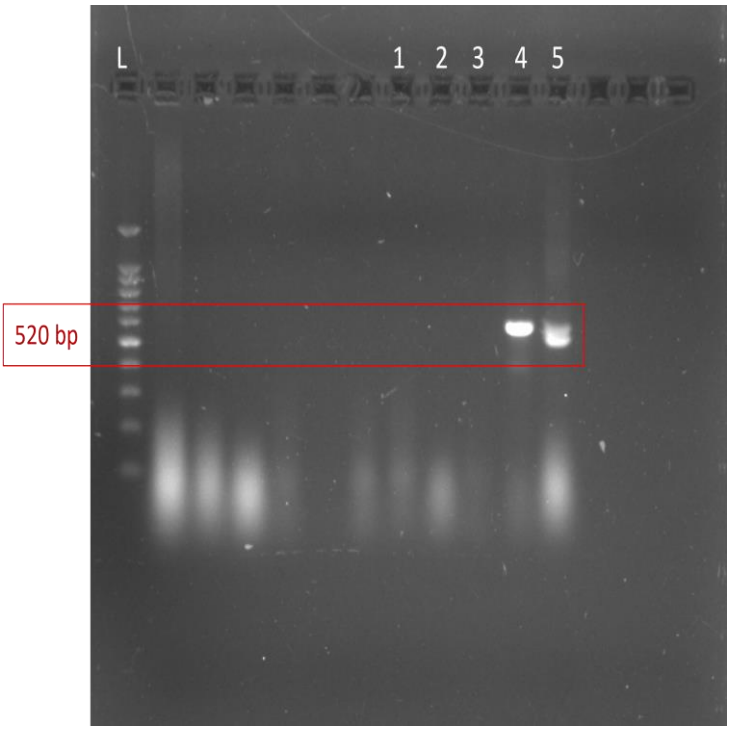

Plate 13. 1.2 % agarose gel profile of DNA samples amplified using DENG primer. L- 100 bp DNA marker; 1- Seed coat; 2- Endosperm; 3- Germ tube; 4, 5- Positive control

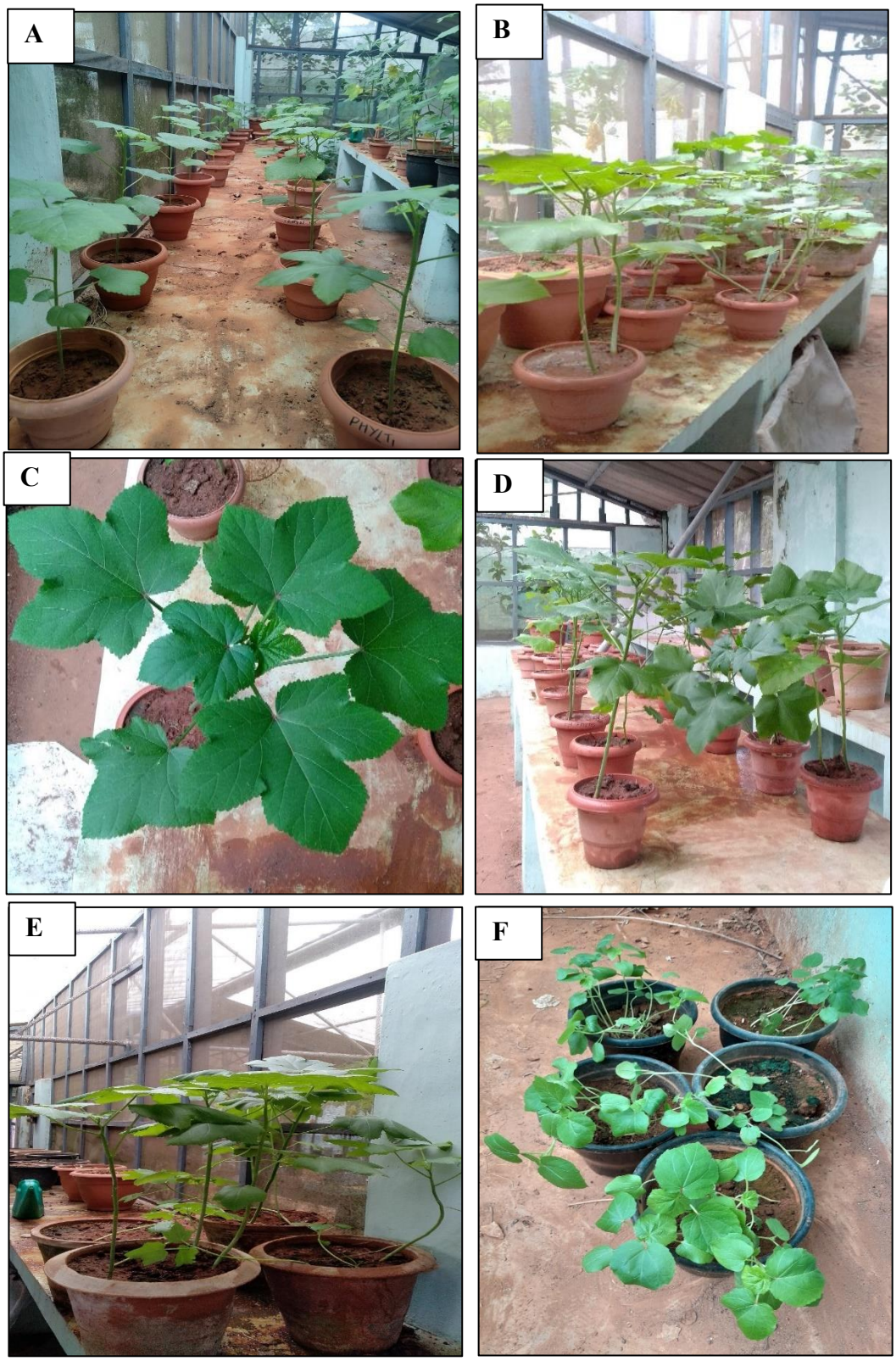

Plate 14. Grow out test of seeds collected from BYVMD infected plants. A, B, C, D-Anjitha; E- Anakomban; F-Varsha Uphar

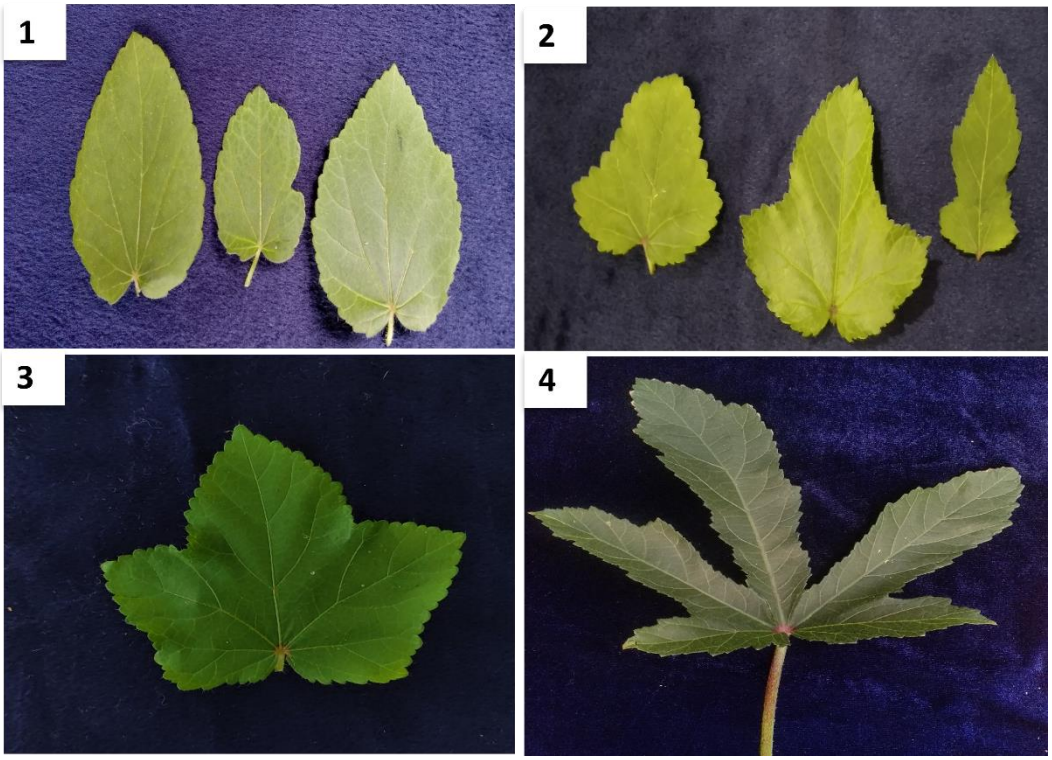

Plate 15. Grow out test plant leaves. 1- Leaves 20 DAS; 2- Leaves 30 DAS; 3- Leaf 60 DAS; 4- Leaf 90 DAS

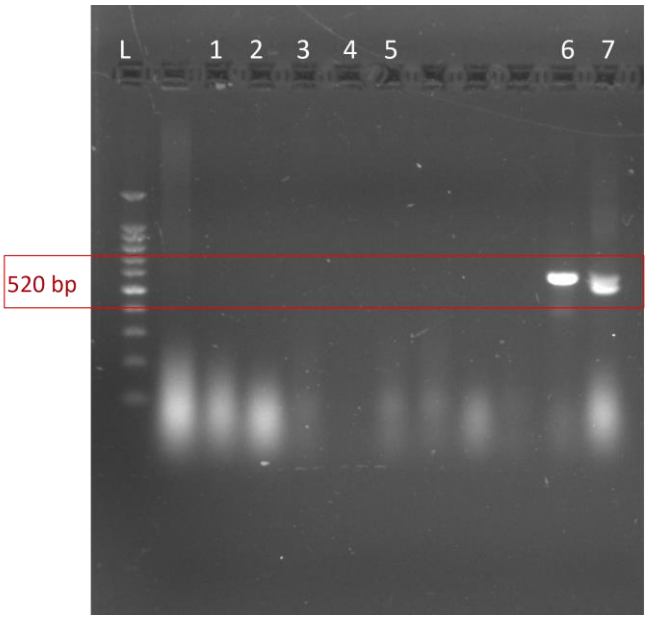

Plate 16. 1.2 % agarose gel profile of DNA samples amplified using DENG primer. L- 100 bp DNA marker; 1- Leaf 20 DAS; 2- Leaf 30 DAS; 3- Leaf 60 DAS; 4- Leaf 90 DAS; 5- leaf of grow out plant in TC; 6,7- positive control

4.3.4 Serological detection of virus in seeds and leaves

DAS- ELISA was done using Polyclonal antisera ToLCNDV New Delhi (DSMZ, Germany) for detecting the presence of virus in random seeds and leaf samples of seedlings raised from seeds collected from infected plants along with healthy control. Healthy and diseased samples of both seed and grow out plant leaves were on par (Table 19). Virus was absent in seed and grow out plant leaves.

Table 19: Reaction of seeds and grow out test leaves in DAS - ELISA

Sl. No	Sample	Antiserum	Absorbance value at 415 nm (mean of 3 replication)		Ratio of absorbance value at 415 nm	Remarks
			Diseased	Healthy		
1	Seed	ToLCNDV *	0.039	0.042	0.928	On par
2	Grow out test leaves	ToLCNDV	0.023	0.020	1.15	On par

*ToLCNDV- *Tomato leaf curl New Delhi virus*

4.4 SCREENING OF DIFFERENT GENOTYPES OF OKRA FOR BYVMV RESISTANCE BY GRAFT TRANSMISSION

Fifteen okra varieties were artificially screened for BYVMD genotypic resistance through wedge grafting. The BYVMD symptoms were visible on the emerging lateral branches on root stock from 8 to 15 days after grafting (Plate 17-18). Disease incidence varied between 33.33% to 100% whereas, vulnerability index varied between 13.34 to 80. Disease incidence, vulnerability index and number of days taken for disease establishment which varies according to the resistance of the genotype is recorded in table 5. Least disease incidence and vulnerability index of 33.33 % and 13.34 respectively was recorded in Phule Vimutha variety. Anakomban and IC 093688 varieties were found to be very

susceptible to BYVMD, with high VI value (80%) and disease incidence (100%). Symptom expression started in Anakomban variety within 8 days where as it took 11 days in IC 093688 accession. IC 00780 took 15 days for symptom development. Only mild vein clearing (mosaic) symptom observed in Anjitha and Phule Vimukta. The varieties were graded for BYVMV resistance following the category chart of table 5. Among 15 varieties screened, 13 varieties were highly susceptible to BYVMD. Anjitha variety was moderately susceptible and Phule Vimukta variety was moderately resistant. Graft union was obtained in 14 plants out of 45 grafted plants (31.11%) (Table 20).

Table 20. Screening of different genotypes of okra for BYVMD resistance by graft transmission

Sl. No.	Treatments	Variety/ Accession no.	Disease incidence (%)	Vulnerability index	Maximum score observed	Days taken for symptom development	Category
1.	T1	IC 052303	100	60	3	11	HS
2.	T2	IC 00780	100	60	3	15	HS
3.	T3	IC 588166	100	60	3	8	HS
4.	T4	IC 002134	100	60	3	8	HS
5.	T5	IC 006101	100	60	3	11	HS
6.	T6	IC 002024	100	60	3	8	HS
7.	T7	IC 043279	100	73.34	4	8	HS
8.	T8	IC 093771	100	73.34	4	8	HS
9.	T9	IC 093688	100	80	4	11	HS
10.	T10	IC 045820	100	73.34	4	9	HS
11.	T11	Anakomban	100	80	4	8	HS
12.	T12	Arka Anamika	100	73.34	4	9	HS
13.	T13	Anjitha	66.66	33.33	3	9	MS
14.	T14	Phule Vimukta	33.33	13.34	2	12	MR
15.	T15	Arka Nikitha	100	60	3	8	HS

*HS- highly susceptible, MS- moderately susceptible, MR- moderately resistant

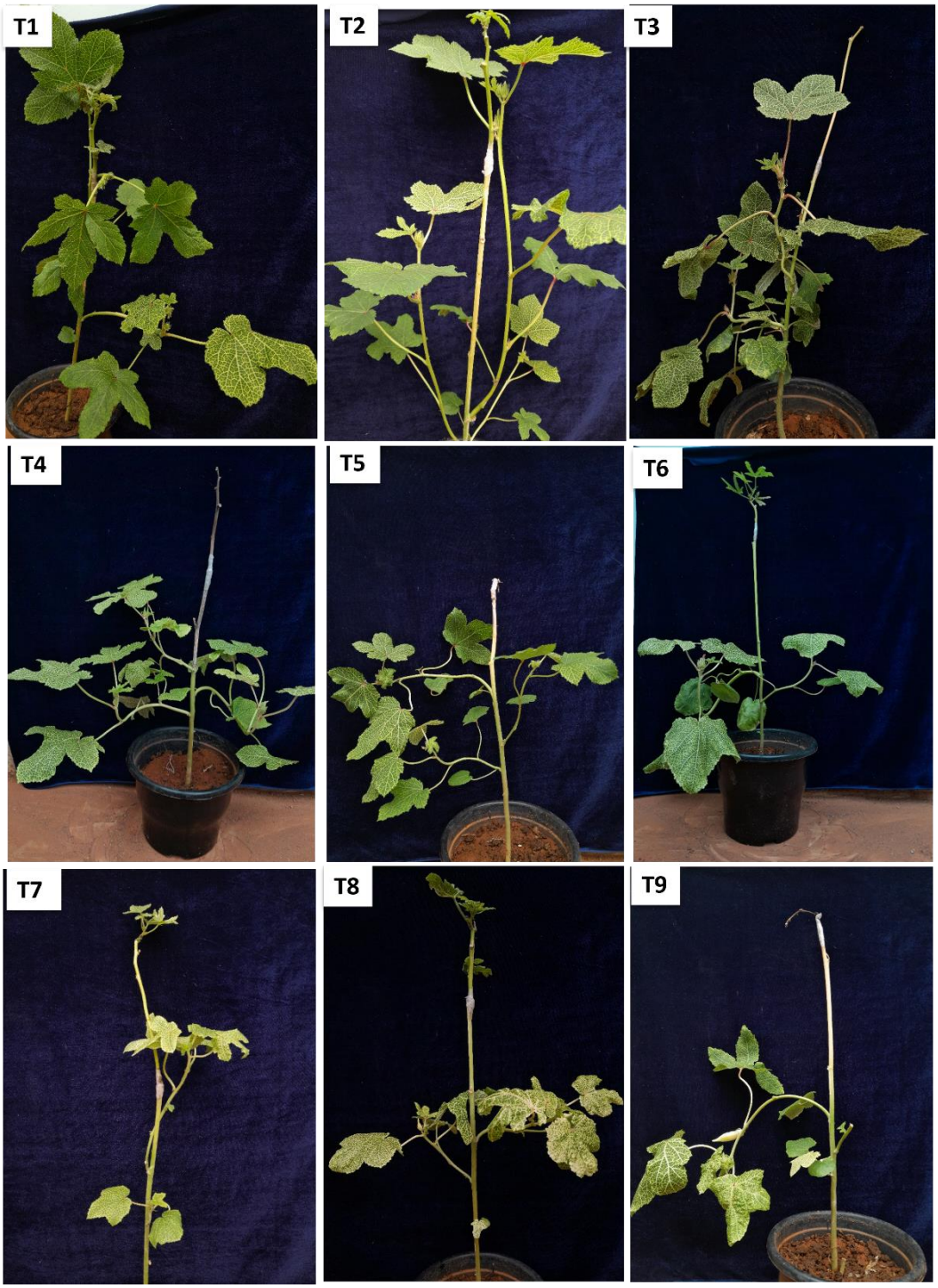

Plate 17. Artificial screening for BYVMD resistance in different accessions. T1- IC 052303; T2- IC 00780; T3- IC 588166; T4- IC 002134; T5- IC 006101;T6- IC 002024; . T7 IC 043279; T8- IC 093771; T9- IC 093688.

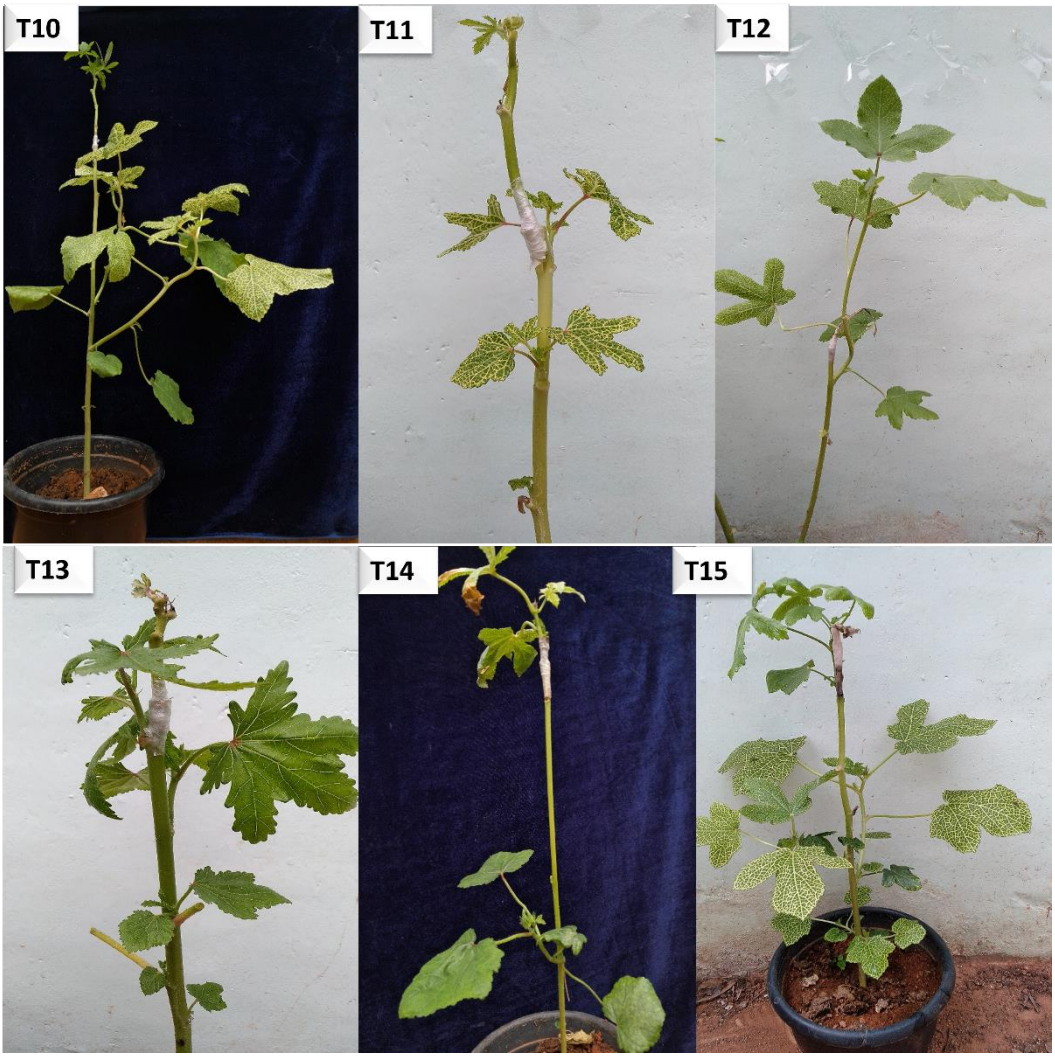

Plate 18. Artificial screening for BYVMD resistance in different varieties. T10- IC 045820; T11- Anakomban; T12- Arka Anamika; T13- Anjitha; T14-Phule Vimukta; T15 Arka Nikitha.

4.5 BIOCHEMICAL ANALYSIS

The biochemical response of different varieties of okra plants to inoculation with the virus by grafting at different days after inoculation was studied based on the activities of defense-related enzymes like peroxidase, polyphenol oxidase, and phenyl alanine ammonia lyase. Enzyme activity was recorded at 30, 45, and 60 days after graft inoculation with the virus. The activity of these enzymes at different growth stages was also analyzed to study the significance of biochemical factors in imparting either resistance or susceptibility reactions in host plants. Enzyme activity was recorded at 30, 45, 75, 90, and 105 days after sowing. The study revealed that the activities of defense-related enzymes varied with genotypes and also with the growth stages of the crop. Tolerant varieties were found to possess the highest content of defense enzymes compared to susceptible varieties.

4.5.1 Peroxidase (PO) assay

Peroxidase activity of leaf samples from graft inoculated plants at all stages of analysis were higher than that of the PO activity of leaf samples collected from uninoculated plants. The PO activity of BYVMD tolerant variety, Phule Vimukta was higher than that of susceptible variety Anakomban at all the crop stages (Table 21). There was a significant difference in the activities of peroxidase enzyme at different crop stages as well as between varieties. The PO activity was found at its peak at 90 DAS (20.07 g^{-1} min^{-1}) in variety, Phule Vimukta, followed by Anjitha (14.44) at 45 DAS. Peroxidase activity of five varieties analysed in 5 different growth stages ranged between 3.38 to 20.07 g^{-1} min^{-1}. Highest PO activity was in Phule Vimukta Variety followed by Anjitha and Arka Anamika. Lowest PO activity was recorded in Anakomban variety. Lowest PO activity was observed in early growth stage that is, at 30 DAS. PO activity of Phule Vimukta at 30 DAS was 17.69 g^{-1} min^{-1} (Table 22).

The peroxidase activity was found to increase on inoculation with the virus. On inoculation with the virus the PO activity increased in both resistant and susceptible variety. At 90 DAS and 45 DAG there was significant increase in the

enzyme activity and thereafter it was found decreasing. PO activity varied between 4.27 to 24.62 g^{-1} min^{-1}. PO activity of 45 DAG is higher than that of 30 DAG and 60 DAG (Table 23).

In statistical analysis of PO enzyme activity of different varieties at various crop growth stages, there was a significant difference with in the factors i.e., varieties and crop stages, as the P value is less than 0.05. There was also significant difference between the factors establishing significant difference in the enzyme activities of varieties over crop growth stages. Statistical analysis of PO enzyme activity in different varieties after graft inoculation with virus also showed significance with P value <0.05. A significant increase in the enzyme activity was observed on inoculation with virus (Table 30 and Table 31).

4.5.2 Polyphenol oxidase (PPO) assay

PPO activity of tolerant variety Phule Vimukta is higher than susceptible variety Anakomban in all growth stages (Table 24). PPO activity of five varieties analysed in 5 different growth stages (30,45,75,90 and 105 DAS) ranged between 0.47 to 3.27 g^{-1} min^{-1}. There was a significant difference in the activities of PPO enzyme at different crop stages as well as between varieties. Highest PPO activity was in Phule Vimukta Variety followed by Anjitha and Arka Nikitha. Lowest PPO activity was recorded in Anakomban variety. PPO activity in different time intervals varied significantly. Highest PPO activity was 90 DAS followed by 75 DAS and 105 DAS. Lowest PPO activity was seen in early growth stage that is, at 30 DAS in all varieties except Phule Vimukta and Anjitha. PPO activity of Phule Vimukta and Anjitha is highest at 30 DAS 3.27 g^{-1} min^{-1} and 2.47 g^{-1} min^{-1} respectively and was found decreasing after 30 DAS. PPO activity of Anakomban, Nikhitha and Arka Anamika were found decreasing from 90 DAS (Table 25).

On analysis of PPO activity after graft inoculation with virus in different varieties at different days after inoculation, significant difference was not observed between the inoculated and uninoculated varieties. PPO activity varied between 1.00 to 3.27g^{-1} min^{-1}. But there was significant difference in PPO activity at

different days after inoculation. PPO activity at 45 DAG was higher than that of 30 DAG and 60 DAG (Table 26).

In statistical analysis of PPO enzyme activity of different varieties at various crop growth stages, there was a significant difference with in the factors i.e., varieties and crop stages, as the P value is less than 0.05. There was also significant difference between the factors establishing significant difference in the enzyme activities of varieties over crop growth stages. Statistical analysis of PPO enzyme activity in different varieties after graft inoculation with virus showed no significance. A significant increase in the enzyme activity was observed in different days after inoculation (Table 30 and Table 31).

4.5.3 Phenylalanine ammonia lyase (PAL) assay

On analysis of PAL activity after graft inoculation with virus in different varieties at different days after inoculation, a decrease in the activity of enzyme after virus inoculation was observed. PAL activity of samples taken from virus inoculated plants was found lower than that of the un inoculated plants. PAL activity was lesser at 30 DAG and 60 DAG and higher at 45 DAG. The PAL activity of BYVMD tolerant variety, Phule Vimukta was higher than that of the susceptible variety, Anakomban in both uninoculated and inoculated conditions in all the three growth stages of analysis. PAL activity varied between 3.7 to 11.32 µg of cinnamic acid g^{-1} fresh weight in different varieties on inoculation with virus (Table 27).

PAL activity of five varieties analysed at 5 different growth stages (30,45,75,90 and 105 DAS) ranged between 1.03 to 22 µg of cinnamic g^{-1}. Highest PAL activity was in Phule Vimukta Variety followed by Anjitha and Arka Nikitha. Lowest PAL activity was recorded in Anakomban variety. PAL activity varied between 3.7 to 8.1 µg of cinnamic acid g^{-1} fresh weight in different varieties. Significant difference was observed in PAL activity at different time intervals also. Highest PAL activity was at 90 DAS followed by 105 DAS. Lowest PAL activity is in early growth stages that is, at 30,45,75 DAS. PAL activity was found

decreasing after 90 DAS (Table 28). PAL activity of different varieties inoculated and uninoculated with virus, analysed at 30,45 and 60 DAG is listed in Table 29.

In statistical analysis of PAL enzyme activity of different varieties at various crop growth stages, there was a significant difference with in the factors i.e., varieties and crop stages, as the P value is less than 0.05. There was also significant difference between the factors establishing significant difference in the enzyme activities of varieties over crop growth stages. Statistical analysis of PAL enzyme activity in different varieties after graft inoculation with virus also showed significance with P value <0.05. A significant decrease in the enzyme activity was observed on inoculation with virus (Table 30 and Table 31).

Table 21 Change in peroxidase activity of susceptible and tolerant variety at different days after graft inoculation with virus

Treatment No.	Treatment	30 DAG**		45 DAG		60 DAG		Total treatment mean
		PO activity* (g^{-1} min^{-1})	% increase over susceptible	P0 activity * (g^{-1} min^{-1})	% increase over susceptible	PO activity* (g^{-1} min^{-1})	% increase over susceptible	
T1	Anakomban variety without graft inoculation	6.18±1.53	_	5.42±1.49	_	4.27±0.61	_	$5.29±0.96^{c}$
T2	Phule Vimukta variety without graft inoculation	19.15±3.65	209.87	20.07±3.14	270.29	17.64±4.50	313.11	$18.95±1.22^{b}$
T3	Anakomban variety with graft inoculation	16.27±2.93	_	18.18±0.85	_	16.33±0.40	_	$16.92±1.08^{b}$
T4	Phule Vimukta variety with graft inoculation	20.95±1.16	28.76	24.62±2.79	35.42	21.45±1.89	31.35	$22.34±1.99^{a}$
	CD value	Treatment: 2.363						
	SE(m)	Treatment: 0.81						

* Mean of three replications ** DAG- Days after grafting of T3 and T4

Table 22. Change in peroxidase activity in different varieties at different growth stages

Treatment No.	Variety	Peroxidase activity* (g^{-1} min^{-1})					Total treatment mean
		30 DAS**	45 DAS	75 DAS	90 DAS	105 DAS	
T1	Anakomban	4.62±0.49	4.98±0.63	6.18±1.53	5.42±1.55	4.27±0.61	$5.09±0.74^{d}$
T2	Arka Anamika	4.35±0.96	7.49±1.28	8.29±0.62	8.47±0.84	7.31±0.49	$7.18±1.66^{bc}$
T3	Anjitha	3.38±1.74	4.95±2.68	10.13±0.64	14.44±1.44	7.91±1.00	$8.16±4.37^{b}$
T4	Phule Vimukta	17.69±5.54	18.91±4.54	19.15±3.65	20.07±3.13	17.64±4.50	$18.69±1.03^{a}$
T5	Arka Nikitha	5.09±1.17	6.64±1.62	5.49±0.64	6.76±0.82	4.44±1.24	$5.68±1.00^{cd}$
	CD value	Treatment - 1.64; Interval - 1.64; Treatment x Interval - 3.67					
	SE(m)	Treatment – 0.577; Interval – 0.577; Treatment x Interval -1.29					

* Mean of three replications ** Days after sowing

Table 23. Change in peroxidase activity of different varieties on graft inoculation with virus at different days interval

Treatment No.	Treatment	Peroxidase activity* (g^{-1} min^{-1})			Total treatment mean
		30 DAG**	45 DAG	60 DAG	
T1	Anakomban uninoculated	6.18±1.53	5.42±1.55	4.27±0.61	$5.29±0.96^{f}$
T2	Arka Anamika uninoculated	8.29±0.62	8.47±0.84	7.31±0.49	$8.02±0.62^{f}$
T3	Anjitha uninoculated	10.13±0.64	14.44±1.44	7.91±1.00	$10.83±3.32^{e}$
T4	Phule Vimukta uninoculated	19.15±3.65	20.07±3.13	17.64±4.50	$18.95±1.22^{bc}$
T5	Arka Nikitha uninoculated	5.49±0.64	6.76±0.82	4.44±1.24	$5.56±1.16^{f}$
T6	Anakomban inoculated	16.27±2.93	18.18±0.84	16.33±0.40	$16.92±1.08^{cd}$
T7	Arka Anamika inoculated	16.54±1.18	17.34±4.84	11.17±0.55	$15.02±3.36^{d}$
T8	Anjitha diseased inoculated	19.23±0.42	22.29±8.13	18.57±7.83	$20.03±1.98^{ab}$
T9	Phule Vimukta inoculated	20.95±1.17	24.62±2.79	21.45±1.90	$22.34±1.98^{a}$
T10	Arka Nikitha inoculated	12.13±3.66	14.66±4.72	8.56±1.30	$11.78±3.06^{e}$
	CD value	Treatment = 2.75; Interval = 1.51			
	SE(m)	Treatment = 0.971; Interval = 0.532			

* Mean of three replications ** DAG- Days after grafting of T5-T10

Table 24. Change in PPO activity of susceptible and tolerant variety at different days after graft inoculation with virus

Treatment No.	Treatment	30 DAG**		45 DAG		60 DAG		Total treatment mean
		PPO activity* (g^{-1} min^{-1})	% increase over susceptible	PP0 activity * (g^{-1} min^{-1})	% increase over susceptible	PPO activity* (g^{-1} min^{-1})	% increase over susceptible	
T1	Anakomban variety without graft inoculation	1.63±0.27	_	2.53±1.82	_	1.2±0.87	_	1.55±0.32
T2	Phule Vimukta variety without graft inoculation	2.11±0.63	29.45	3.27±2.65	29.25	1.47±0.42	22.5	2.07±0.59
T3	Anakomban variety with graft inoculation	1.87±1.86	_	3.27±2.47	_	1.00±0.84	_	1.73±0.82
T4	Phule Vimukta variety with graft inoculation	2.20±0.61	17.65	3.40±2.83	3..90	2.13±1.27	113	2.10±0.78
	CD value	Time interval- 0.684						
	SE(M)	Time interval- 0.234						

* Mean of three replications ** DAG- Days after grafting of T3 and T4

Table 25. Change in PPO activity in different varieties at different growth stages

Treatment No.	Variety	PPO activity* (g^{-1} min^{-1})					Total treatment mean
		30 DAS**	45 DAS	75 DAS	90 DAS	105 DAS	
T1	Anakomban	0.47±0.30	0.73±0.35	1.62±0.27	1.82±0.64	1.20±0.87	$1.17±0.57^{c}$
T2	Arka Anamika	1.04±0.41	0.71±0.88	1.56±0.14	2.04±0.10	1.20±0.20	$1.31±0.51^{c}$
T3	Anjitha	2.47±0.35	1.98±1.10	1.70±0.58	1.91±0.63	1.22±0.63	$1.86±0.45^{b}$
T4	Phule Vimukta	3.27±0.24	2.53±1.04	2.11±0.63	2.65±0.56	1.47±0.42	$2.40±0.66^{a}$
T5	Arka Nikitha	0.97±0.51	1.56±0.58	1.82±0.20	2.24±0.43	1.31±0.20	$1.57±0.49^{bc}$
	CD value	Treatment = 0.41; Interval = 0.41; Treatment x Interval = 0.92					
	SE(M)	Treatment =0.145 ; Interval =0.145 ; Treatment x Interval = 0.323					

* Mean of three replications ** Days after sowing

Table 26. Change in PPO activity of different varieties on graft inoculation with virus at different days interval

Treatment No.	Treatment	PPO activity* (g^{-1} min^{-1})			Total treatment mean
		30 DAG**	45 DAG	60 DAG	
T1	Anakomban uninoculated	1.62±0.27	1.82±0.64	1.2±0.87	1.55±0.32
T2	Arka Anamika uninoculated	1.68±0.71	1.99±0.40	1.28±0.42	1.65±0.36
T3	Anjitha uninoculated	1.98±0.54	2.41±0.77	1.33±0.47	1.91±0.54
T4	Phule Vimukta uninoculated	2.11±0.63	2.65±0.56	1.49±0.43	2.08±0.58
T5	Arka Nikitha uninoculated	1.75±0.11	2.02±0.39	1.63±0.12	1.80±0.20
T6	Anakomban inoculated	1.87±1.86	2.47±0.74	0.84±0.21	1.73±0.82
T7	Arka Anamika inoculated	1.94±0.23	2.30.28	0.98±0.33	1.74±0.68
T8	Anjitha diseased inoculated	2.13±0.54	2.67±0.96	1.12±0.41	1.97±0.79
T9	Phule Vimukta inoculated	2.20±0.61	2.82±0.73	1.27±0.86	2.10±0.78
T10	Arka Nikitha inoculated	2.02±0.11	2.51±0.58	1.84±0.85	2.12±0.35
	CD value	Time interval- 0.33			
	ME(S)	Time interval- 0.119			

* Mean of three replications ** DAG- Days after grafting of T5-T10

Table 27. Change in PAL activity (µg of cinnamic acid g^{-1} fresh weight) of susceptible and tolerant variety at different days after graft inoculation with virus

Treatment No.	Treatment	30 DAG**		45 DAG		60 DAG		Total treatment mean
		PAL activity*	% increase over susceptible	PAL activity *	% increase over susceptible	PAL activity*	% increase over susceptible	
T1	Anakomban variety without graft inoculation	1.30±0.20	_	12.40±1.30	_	2.92±0.42	_	5.54±5.99^{bc}
T2	Phule Vimukta variety without graft inoculation	3.40±0.75	161.53	22.00±5.00	77.42	8.57±1.20	193.49	11.32±9.60^{a}
T3	Anakomban variety with graft inoculation	2.10±0.60	_	7.48±0.16	_	1.55±0.31	_	3.71±3.28^{c}
T4	Phule Vimukta variety with graft inoculation	8.20±7.95	290.48	7.57±1.17	1.20	4.27±0.47	175.48	6.68±2.11^{b}
	CD value	Treatment = 2.73 Interval = 2.37 Treatment x Interval = 4.73						
	SE(m)	Treatment = 0.94; Interval = 0.81; Treatment x Interval = 1.62						

* Mean of three replications ** DAG- Days after grafting of T3 and T4

Table 28. Change in PAL activity of different varieties in different growth stages

Treatment	Variety	PAL activity* (µg of cinnamic acid g^{-1} fresh weight)					Total treatment mean
		30 DAS**	45 DAS	75 DAS	90 DAS	105 DAS	
T1	Anakomban	1.03±0.25	1.20±0.40	1.30±0.20	12.40±1.30	2.92±0.42	3.77±4.63^{c}
T2	Arka Anamika	1.33±0.50	1.47±0.21	1.80±0.26	14.00±0.69	3.63±0.35	4.45±5.17^{c}
T3	Anjitha	2.00±0.95	2.00±0.44	2.73±0.80	17.30±0.56	6.83±2.08	6.17±6.36^{b}
T4	Phule Vimukta	3.20±0.46	3.37±0.32	3.40±0.75	22.00±5.00	8.57±1.20	8.11±7.96^{a}
T5	Arka Nikitha	2.40±0.17	2.7±0.87	2.4 ±0.60	14.37±1.76	5.43±0.75	5.47±5.11^{b}
CD value		Treatment = 0.95; Interval = 0.95; Treatment x Interval = 2.12					
SE(m)		Treatment = 0.33 ; Interval =0.33 ; Treatment x Interval = 0.74					

* Mean of three replications ** Days after sowing

Table 29. Change in PAL activity of different varieties on graft inoculation with virus at different days interval

Treatment No.	Treatment	PAL activity* (µg of cinnamic acid g^{-1} fresh weight)			Total treatment mean
		30 DAG**	45 DAG	60 DAG	
T1	Anakomban uninoculated	1.30±0.2	12.40±1.3	2.92±0.42	5.54±5.99^{de}
T2	Arka Anamika uninoculated	1.73±0.67	14.23±0.59	3.63±0.38	6.53±6.74^{cd}
T3	Anjitha uninoculated	3.13±0.46	17.53±2.12	6.77±1.01	9.14±7.49^{b}
T4	Phule Vimukta uninoculated	3.40±0.75	22.00±5.00	8.57±1.20	11.32±9.60^{a}
T5	Arka Nikitha uninoculated	2.87±0.45	15.17±0.71	3.97±0.90	7.33±6.80^{c}
T6	Anakomban inoculated	2.10±0.62	7.48±0.16	1.55±0.31	3.71±3.28^{f}
T7	Arka Anamika inoculated	4.27±0.68	6.80±0.35	1.60±0.56	4.22±2.60^{ef}
T8	Anjitha diseased inoculated	7.53±1.16	7.13±0.71	2.53±0.30	5.73±2.78 ^{cde}
T9	Phule Vimukta inoculated	8.20±7.95	7.57±1.17	4.27±0.47	6.68±2.11 ^{cd}
T10	Arka Nikitha inoculated	4.57±0.70	5.30±0.62	2.23±0.50	4.03±1.60^{ef}
	CD value	Treatment-1.78; Interval = 0.97; Treatment x Interval = 3.08			
	SE(m)	Treatment-0.63; Interval =0.34; Treatment x Interval = 1.88			

* Mean of three replications ** DAG- Days after grafting of T5-T10

Table 30. ANOVA analysis of PO, PPO, and PAL activities in different varieties at different growth stages

Source of variation	PO Activity				PPO Activity				PAL activity			
	DF	MSS	F Value	P Value	DF	MSS	F Value	P Value	DF	MSS	F Value	P Value
Factor A*	4	465.734	93.248	0.00	4	3.587	11.439	0.000	4	42.408	25.471	0
Factor B**	4	35.116	7.031	0.00	4	1.515	4.832	0.002	4	540.214	324.473	0
A X B	16	9.595	1.921	0.04	16	0.726	2.315	0.012	16	5.799	3.483	0
Error	50	4.995	NA	_	50	0.314	_	_	50	1.665	_	_

* Different varieties ** Growth stage of plant

Table 31. ANOVA analysis of PO, PPO, and PAL activities in different varieties on graft inoculation with virus at different days interval

Source of variation	PO Activity				PPO Activity				PAL activity			
	DF	MSS	F Value	P Value	DF	MSS	F Value	P Value	DF	MSS	F Value	P Value
Factor A*	9	335.126	39.474	0.000	9	0.365	0.865	0.561	9	51.480	14.510	0
Factor B**	2	49.799	5.866	0.005	2	8.647	20.482	0.000	2	593.756	167.351	0
A X B	18	10.974	1.293	0.226	18	0.171	0.404	0.982	18	36.751	10.358	0
Error	60	8.490	_	_	60	0.422	_	_	60	3.548	_	_

* Varieties with or without virus inoculation ** Days after graft inoculation

4.6 MERISTEM CULTURE FOR THE PRODUCTION OF VIRUS-FREE PLANTS

Meristem culture of okra was standardised using meristems collected from *in vitro* established okra plants from seeds and from infected plant shoots. Production of virus free plants were done from meristem collected from infected plant shoots and seeds of BYVMV infected plants.

3.6.1 Meristem culture standardization

Meristem culture of okra was standardized using meristems collected from *in vitro* established okra plants from seeds and infected shoots. Surface sterilization of the seeds by treating with 0.2% mercuric chloride for 1 minute was more effective as the contamination percentage was zero. The higher concentrations of mercuric chloride resulted in decrease in germination of seeds. Different concentration of mercuric chloride and percentage contamination of seeds are given in Table 32.

MS media supplemented with 0.5µM benzyl aminopurine + 0.1 µM Naphthalene acetic acid + 0.1µM Gibberellic acid (Nair *et al*., 1979) was more effective for meristem establishment with 100 % regeneration of meristem (Table 33). Multiple shoots were initiated in all the meristems kept in MS media with 1.0 mg/l BAP + 0.5 mg/l GA_3 (Table 34). Out of ten shoots kept in MS media with 0.1 mg/l BAP + 0.02 mg/l GA_3, root was initiated in three shoots. No root initiation was obtained in MS media with 1.0 mg/l IBA + 1.0 mg/l BAP + 0.02 mg/l GA_3 (Table 35).

4.6.2 Meristem culture from infected plant's seed and shoot

Seeds of BYVMV infected plants were used for production of virus free plants. The germination percentage was 53.33 %. Five meristems were established successfully from the eight meristems kept. Multiple shoots were developed on all the five meristems kept in shoot initiation media (Table 36) Three shoots developed roots in root initiation and proliferation media. Rooted plants were acclimatized in coir pith compost with 1 % MS supplement spray (Plate 19).

Table 32. Effect of different concentration of mercuric chloride as surface sterilant

Sl. No.	Mercuric chloride (%) for 1 min	No. of seeds kept	No. of seeds germinated	Germination percentage	No. of contaminated seeds (out of total seed)	Contamination percentage
1.	0.05	10	7	70	9	90
2.	0.1	10	6	60	5	50
3.	0.2	10	6	60	0	0
4.	0.3	10	3	30	0	0

Table 33. Effect of different media on meristem pre- establishment media

Sl. No.	Media	No of meristem kept	No. of Regenerated meristem	Regeneration percentage
1.	Half M S media	5	2	40
2.	M S media	5	2	40
3.	Hormonal media (Nair *et al.*, 1979)	5	5	100

Table 34. Effect of different media on shoot multiplication

Sl. No.	Media	No. of established meristem kept	No. of plants with multiple shoots	No. of shoots initiated (mean)	Regeneration percentage
1.	MS media with 5µM BAP + 0.1 µM NAA+ 0.1µM GA_3 (Nair *et al*., 1979)	3	0	0	0
2.	MS media with 1.0 mg/l BAP + 0.5 mg/l GA_3 (Anisuzzaman *et al.*,2010)	3	3	6	100
3.	MS media with 1.0 mg/l IBA and 1.0 mg/l BAP + 0.02 mg/l GA_3	3	0	0	0

Table 35. Effect of different media on root induction

Sl. No.	Media	No of shoots kept	No. of plants rooted
1.	MS media with 0.1 mg/l BAP + 0.02 mg/l GA_3	10	3
2.	MS media with 1.0 mg/l IBA + 1.0 mg/l BAP + 0.02 mg/l GA_3	5	0

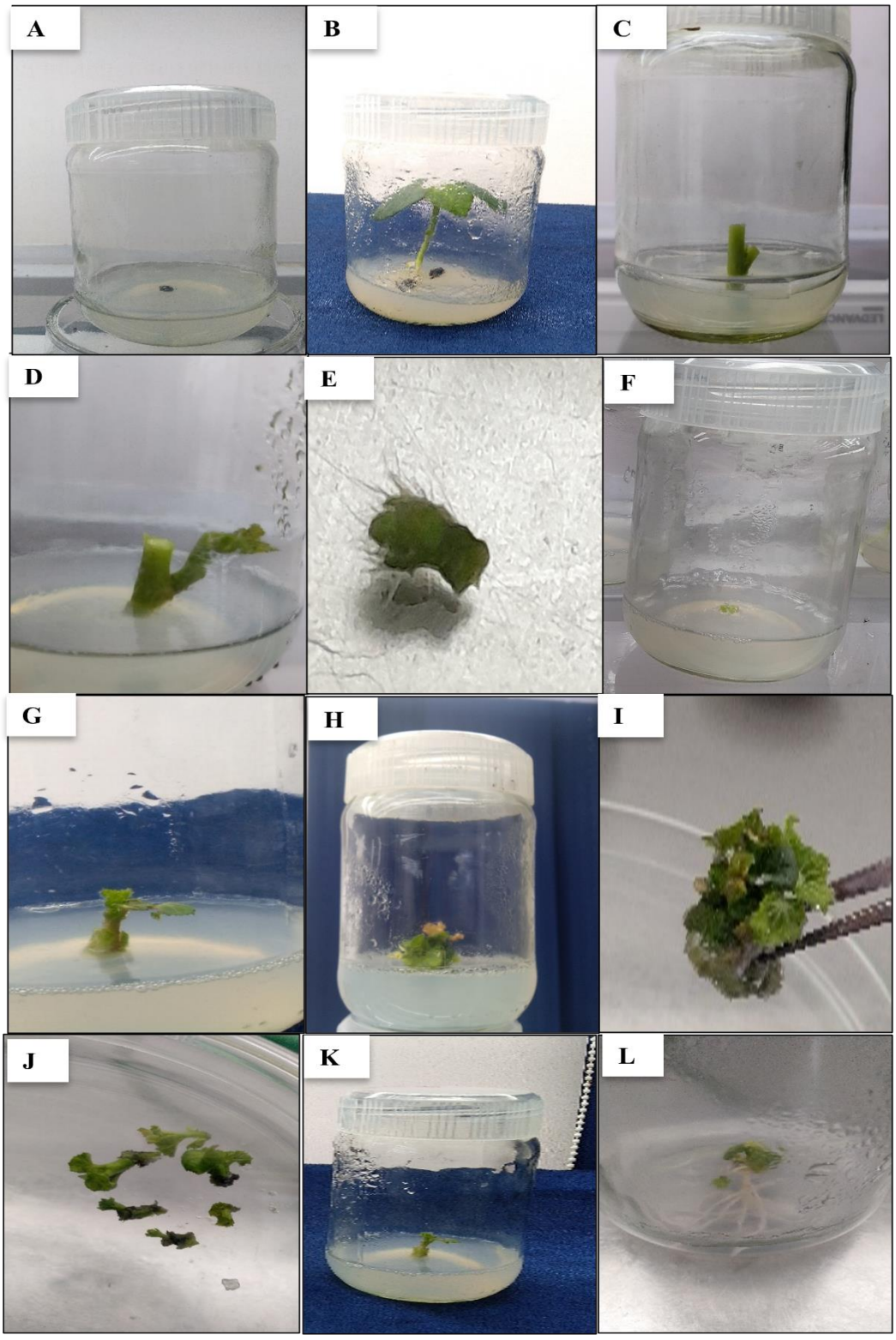

Plate 19. Meristem culture. A- Seed placed in half MS media; B- Seedling; C- shoot in half MS media; D- lateral shoot growing; E- Meristematic portion separated from plant; F- Meristem kept in hormonal media; G- Meristem regenerated; H,I- Multiple shoot initiation from regenerated; J- Shoots separated; K- Shoot kept in rooting media; L- Root initiation and proliferation.

Table 36. Meristem culture from shoots and seeds of infected plants

Sl. No.	Explant (from BYVMV infected plant)	No. of explant		No. of meristem		No. of meristem kept for shoot initiation	No. of multiple shoots initiated
		Placed	Regenerated	Placed	Regenerated		
1.	Seeds	15	8	8	5	5	4-6
2.	Shoots	20	20	20	11	11	4-6

4.6.3 Virus indexing

Amplicon was absent in PCR product of DNA extracted from meristem cultured plants. Thus, confirmed that the virus was absent in meristem established plants (Plate 20).

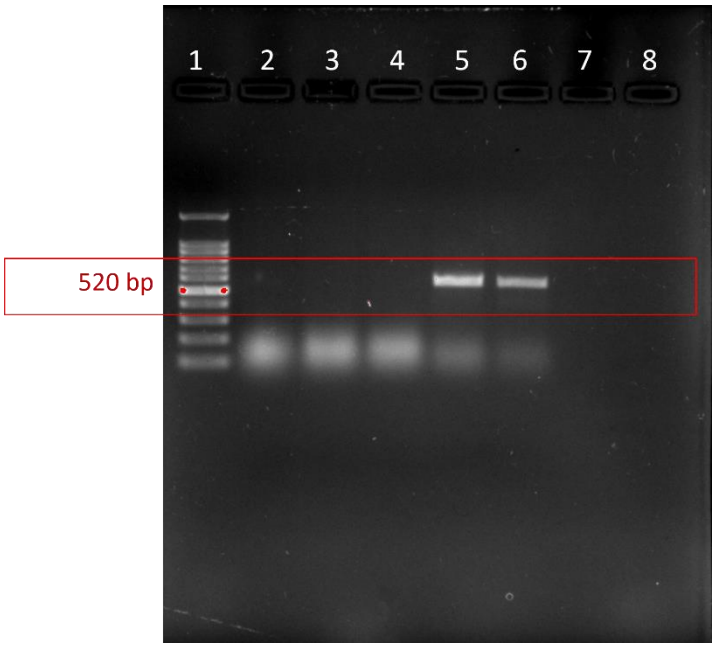

Plate 20 :1.2 % agarose gel profile of DNA samples amplified using DENG primer 1. 100 bp DNA marker 2,3,4. Meristem cultured plants 5,6. Positive control

Discussion

5. DISCUSSION

The research work entitled 'Biochemical response of okra to *Bhendi yellow vein mosaic virus* and production of virus free plants*'* is done for studying the host pathogen interaction in *Bhendi yellow vein mosaic virus* (BYVMV) infected okra, screening of different genotypes of okra cultivars for disease resistance and production of virus free plants using meristem culture. The findings of research are discussed in detail in this chapter.

5.1 COLLECTION AND MAINTENANCE OF VIRUS INFECTED PLANTS

Survey was done in Thrissur (AEU 10- North central laterites), Thiruvananthapuram (AEU 8- Southern laterites) and Palakkad districts (AEU 10- North central laterites) for finding various symptoms, disease incidence and vulnerability index of BYVMD. Symptoms observed in various varieties were similar and some varieties showed difference in symptom in different locations. Mottling, vein clearing, vein thickening and bleaching of fruits were common in all locations. An exceptional symptom of decrease in internode length, increase in leaf size along with long petiole and floral deformity was found in Anjitha variety cultivated in Department of vegetable science in College of Agriculture, Vellayani, Thiruvananthapuram (AEU 8). Disease incidence was high when the plants enter into flowering stage. The per cent okra yellow vein mosaic disease incidence in the various BYVMV isolates varied from 37.20 to 100. Whereas vulnerability index of various BYVMV isolates varied from 23.40 to 85.20. Maximum score of vulnerability index was 5 for all surveyed locations. There was a considerable drop in the amount of vegetable produced, along with smaller leaves and fruits in surveyed locations. Reduction in yield is a result of reduction in photosynthetic area (Pun and Doraiswamy, 1999).

Cultivated varieties include Anakomban, Anjitha, Arka Anamika and Varsha Uphar. Highest okra yellow vein mosaic disease incidence of 100% was observed in Anakomban and Varsha Uphar variety whereas vulnerability index of these varieties were 83.53 and 80 respectively.

Highest vulnerability index was in Arka Anamika variety cultivated in Vellayani, Thiruvananthapuram. Lowest vulnerability index (37.20) and disease incidence (23.4 %) were observed in Anjitha variety.

Chippy (2020) conducted survey in three fields of Thiruvananthapuram district (Vellayani, Pappanchani, and Palappur) and reported 67 to 100% disease incidence and 32.60 to 71.07 vulnerability index of BYVMD. Sixty-seven per cent disease incidence and 32.60 vulnerability index were recorded in Anjitha variety cultivated in Vellayani.

Per cent disease incidence of 91.81 for Arka Anamika variety was noticed in the field. The variety, Arka Anamika, which is a hybrid derivative selection obtained from an interspecific cross between *A. esculentum* and *A. tetraphyllus*, was previously reported to be resistant to BYVMV (Borah *et al*., 1992) was observed susceptible with a vulnerability index of 85.2, which indicates the breakdown of resistance in this variety.

Hundred per cent BYVMD incidence was observed in the variety, Varsha Upahar, cultivated in Vellanikkara (Thrissur). Varsha Uphar developed by HAU, Hisar from a cross between Lam Selection IX and Parbhani Kranti reported with resistance to okra yellow vein mosaic geminivirus was found to be highly susceptible with 100% disease incidence and a V.I. of 80. Arka Anamika and Varsha Uphar varieties were reported as tolerant to *Okra yellow vein mosaic virus* (Kolakar *et al*., 2018).

The virus was maintained by graft transmission in insect proof glass house for further studies. Infected scions were wedge grafted on to susceptible root stock of Anjitha and Anakomban varieties and showed 87.50 per cent disease transmission. The disease symptoms appeared in the newly emerged leaves of root stock after 8-9 days of graft inoculation. The yellow vein mosaic disease (YVMD) was distinguished by indications of a uniform intertwining network of yellow veins and islands of green tissue. In its initial stages of infection, leaves displayed solely the yellowing of veins and veinlets, though subsequently, the entire leaf underwent

complete yellowing and bleaching. Graft union was not observed in all plants. The symptoms were similar to that observed in field condition. Naveen (2018) and Bincy (2019) have also reported successful graft transmission of geminivirus in okra with 70 % and 100% transmission respectively. Similar observation was noticed in this study also. A slight variation in the graft transmission percentage may be due to the difference in susceptibility of the rootstock. Here, out of the two varieties used for graft transmission, Anakomban variety took only 8 days and Anjitha variety took 9 days in an average for disease development after graft inoculation. Also in the field observations, Anjitha was found to be with less disease incidence and V.I. than Anakomban cultivar of okra.

5.2 MOLECULAR AND SEROLOGICAL DETECTION

The total DNA from infected plant samples were isolated using modified CTAB method. Increasing the quantity of CTAB buffer used for extraction by ten times was effective to manage the mucilaginous nature of okra leaf sample due to the presence of polysaccharides in it. The DNA/ RNA ratio measured using nanodrop spectrophotometer value varied between 1.80 to 2.1. Leaf samples showing two different symptoms were taken for DNA isolation. Leaves showing vein clearing as well as leaves showing enation symptoms were taken for the study. Leaf samples of the weed species, *Ageratum*, was also taken for molecular detection. The quality of extracted DNA was also accessed by running in 0.8 % agarose gel. PCR amplification of DNA isolated from both the infected leaf samples using the DENG primer (universal primer for begomoviruses) yielded an expected amplicon of size 520 bp. The PCR product was electrophoresed in 1.2 % molecular grade agarose gel. Bands without smear and high thickness indicated the good quality and quantity of the extracted DNA.

BLAST analysis of the sequences of amplicon obtained from okra leaf samples with vein clearing and enation symptom showed 96.27 and 96.67 per cent similarity with DNA-A segment of bhendi yellow vein mosaic virus. The sequence of amplicon from *Ageratum conyzoides* with virus disease symptom showed 93.99

per cent homology with french bean leaf curl Madkeri virus isolate FB1 pre-coat protein (AV2), coat protein (AV1). 89.46 per cent homology with ageratum yellow vein Sri Lanka virus segment DNA-A and ageratum yellow vein Sri Lanka virus segment DNA-A followed by 88.29 per cent similarity with passion fruit leaf curl Madkeri virus isolate PF1 segment DNA-A pre-coat protein (AV2), coat protein (AV1). Naveen (2016) reported 87% sequence similarity of amplicons obtained from yellow vein symptomatic *A. conyzoides* collected from BYVMD infected field with ageratum yellow vein Sri Lanka virus (Accession. No. AF314144.1).

ELISA and DIBA are two effective ways for the detection of presence of virus. The infected samples were subjected to serological detection through the utilization of both DAS - ELISA and DIBA techniques. DAS- ELISA using polyclonal antisera for ToLCNDV New Delhi virus (DSMZ, Germany) confirmed the presence of virus in leaf samples with BYVMD symptom. Several researchers have previously documented the immunological detection of BYVMV through the employment of DAS and DAS ELISA technique using ToLCNDV antiserum. Absorbance at 415 nm was 0.17 and 0.007 for diseased and healthy leaf samples respectively. Diseased leaf samples showed 24.29 fold increase in absorbance over the healthy samples at 415 nm. Similar result was got for studies by Bincy (2019) where DAS-ELISA using polyclonal antiserum specific to *Tomato leaf curl New Delhi virus* (ToLCNDV) yielded more absorbance at 415 nm in infected samples than the healthy samples.

DIBA was also done using polyclonal antisera of ToLCNDV (DSMZ, Germany) for detecting the presence of virus in leaf samples showing vein clearing along with healthy and buffer control. It was observed that all the test samples with BYVMD symptom exhibited positive reactivity during the DIBA detection, thereby establishing the presence of *Begomovirus*. Healthy and buffer control showed negative values which indicate the absence of virus in it. Diseased sample showed 37.47% increase of volume intensity than the negative control.

5.3 SEED TRANSMISSION STUDIES

Knowledge on seed transmission of diseases is very important as the disease severity will be high when the pathogen infects the plant at its early stage. Various methods were followed to check the disease transmission through seeds. Two hundred and eighty six seeds collected from BYVMV infected plants were sown in pots and maintained in insect proof glasshouse condition for 4 months. Among the 154 germinated, none of the plants showed BYVMV symptoms. Similar result was concluded by Bincy (2019) by conducting grow out test for 10 seeds in insect proof condition for 2 months. Four hundred seeds of BYVMV infected okra tested by Sisodia and Mahatma (2020) didn't show any type of symptoms and none of the plants tested showed presence of positive reaction in the PCR. In the present investigation also none of the plants of grow out test exhibited symptoms. Asymptomatic leaves from random seedlings from the grow out test plants were examined for the presence of virus by PCR using universal primer for geminiviruses. On PCR detection of samples taken from plants at various dates after sowing also showed the absence of amplicon confirming the absence of seed transmission of the virus.

Seeds collected from BYVMD infected plants were also grown in tissue culture media to ensure complete absence of vector. The PCR analysis of the DNA extracted from leaves collected from *in-vitro* grown plants also showed the absence of amplicon.

For a more conclusive result, the presence of virus in seed was checked on seed parts *viz.*, seed coat, endosperm and germ tube as well as whole seed using molecular and serological techniques. Presence of virus was detected in 4 seeds out of 20 seeds examined. Thus, the virus presence was detected in 20 % of seeds examined by PCR. An expected amplicon of size 520 bp was detected on PCR analysis of DNA isolated from whole seeds using DENG primer. None of the samples of seed parts accessed separately gave a positive result. Thus, it was unable to distinguish in which part of seed, virus is present. Sisodia and Mahatma (2020)

reported 50-80 per cent seeds of infected plant carry the virus. He detected presence of virus in 80%, 60%, 50% of cotyledon, seed coat and embryonic axis respectively by studying 10 seeds of Gujarat Okra-1 variety.

Mahatma and Pawar (2015) reported the presence of *Geminivirus*, *Mungbean Yellow Mosaic virus* in mungbean (*Vigna radiata*) seeds. Virus was present in different parts of the seed (seed coat and cotyledon). However, they could not detect the presence of virus in embryonic axis and gynoecium of the seed and concluded that because of the absence of virus in embryonic axis, virus is non-seed transmitted. Similarly, the absence of virus in the embryonic axis might be the reason for absence of BYVMD in seedlings of seeds collected from infected plants in grow out test. Even though seed transmission could not be proved in this virus, less germination percentage observed in the present study reveals that the quality of seeds is affected by the viral infection of okra plants reducing the germination of seeds and seedling vigor.

DAS-ELISA using ToLCNDV (DSMZ, Germany) was done for the serological detection of virus in seeds and the result was negative as the ratio of absorbance value at 415 nm was on par with the negative control. It was unable to serologically detect the presence of virus in seed. It may be due to the low concentration of virus in seed or due to the absence of virus in random samples of seed.

5.4 SCREENING OF DIFFERENT GENOTYPES OF OKRA FOR BYVMV RESISTANCE BY GRAFT TRANSMISSION

Fifteen okra varieties were screened for BYVMV by grafting. Ten varieties were NBPGR accessions (IC 052303, IC 00780, IC 588166, IC 002134, IC 006101, IC 002024, IC 043279, IC 093771, IC 093688, IC 045820) and five were the popular varieties among farmers (Anakomban, Arka Anamika, Anjitha, Phule Vimukta, Arka Nikitha). Following wedge grafting method for disease transmission helped to nullify the possibility of disease escape and it will provide a conclusive report on the genotypic resistance of the variety. Based on vulnerability index,

screened varieties were classified into five groups such as resistant, moderately resistant, moderately susceptible, susceptible and highly susceptible. Graft union was obtained in 14 plants out of 45 grafted plants (31.11%).

None of the screened varieties is genotypically resistant to BYVMV. The BYVMD symptoms were visible on the emerging lateral branches on root stock from 8 to 15 days after grafting. Disease incidence varied between 33.33% to 100% whereas vulnerability index varied between 13.34 to 80. Thirteen varieties were highly susceptible to BYVMD. Anjitha variety was moderately susceptible and Phule Vimukta variety was moderately resistant to BYVMD.

Least disease incidence and vulnerability index of 33.33 % and 13.34 respectively was recorded in the Phule Vimuka variety. It is a varietry released from Mahatma Phule Krishi Vidyapeeth, Rahuri in 2016 with attractive green colour fruits. It is best for subtropical climate with well drained medium fertile soil. It has a productivity of 200-210 q/ha with 110-120 days crop duration. It was reported as resistant to yellow vein mosaic virus disease. But in this study, it is proved that it is not genotypically resistant to BYVMV. But the possibility of resistance cannot be overruled as there is a chance for field resistance to virus aided by resistance to whitefly. Only mild vein clearing (mosaic) symptom observed in Anjitha and Phule Vimukta.

Anjitha variety released from Kerala Agricultural University in 2006 is developed by interspecific hybridization followed by mutation breeding and selection. It is an early maturing high yielding (14.6t/ha) variety with YVM resistance. In this study, it is clear that Anjitha variety is not genotypically resistant to BYVMV and it is moderately susceptible to BYVMV.

The genotype Arka Anamika, which was previously reported to be resistant to BYVMV (Borah *et al*., 1992), exhibited a pronounced susceptibility to BYVMD with high disease incidence and vulnerability index which conclude the breakdown of resistance in variety. Breakdown of resistance to viral diseases in different genotypes may be a result of distinct interplay between the specific strain of the

virus and the genetic makeup of the plant, or the interaction between the vector and the genotype, or even the modified feeding conditions of the vector (Polston and Anderson,1997).

Anakomban and IC 093688 varieties were found to be very susceptible to BYVMD, with high VI value (80%) and disease incidence (100%). Symptom expression started in Anakomban variety within 8 days whereas it took 11 days in IC 093688 accession. Survey shows that Anakomban variety is very popular among farmers despite it is highly susceptible to BYVMV. Its high yield and less maintenance make it more popular among farmers.

5.5 BIOCHEMICAL ANALYSIS

On screening for BYVMV resistance, Phule Vimukta variety was found to be tolerant to disease and Anakomban as highly susceptible. Analysed the PO, PPO and PAL activity of Phule Vimukta and Anakomban variety and the host resistance mechanism was studied. Biochemical response of resistant and susceptible cultivars was studied based on the analysis of defense related enzymes *viz.,* PO, PPO, and PAL activity. Enzyme activity of Phule Vimukta and Anakomban variety was studied at different crop stages as well as after inoculation with virus and the mechanisms of host resistance was studied. Peroxidase, polyphenol oxidase (PPO), and phenylalanine ammonia-lyase (PAL) are defense related enzymes found in plants, and they play various roles in the plant's response to various stress factors, including plant virus diseases. Specific response of a plant to a viral infection can vary depending on the plant species, the type of virus, and the environmental conditions. The interplay of these enzymes and other defense mechanisms in plants helps them resist and mitigate the damage caused by viral infections. Peroxidases are a class of enzymes that are involved in the detoxification of reactive oxygen species (ROS) in plant cells which are produced as a defense response to various stresses, including viral infections. Peroxidases help neutralize these harmful molecules and prevent oxidative damage to plant tissues.

Peroxidase activity of five varieties analysed in five different growth stages (30,45,75,90 and 105 DAS) ranged between 3.38 to 20.07 g^{-1} min^{-1}. PO activity of moderately resistant variety Phule Vimukta was with 270.29 % and 35.02 % more enzyme activity than the susceptible variety, Anakomban at 90 days after sowing and 45 days after graft inoculation respectively. Lowest PO activity was observed in early growth stage that is, at 30 DAS. Highest PO activity was observed in virus inoculated BYVMD moderately resistant variety Phule Vimukta (24.62 g^{-1} min^{-1}) followed by moderately susceptible variety Anjitha (22.29 g^{-1} min^{-1}) at 45 DAG. Lowest PO activity was recorded in the uninoculated Anakomban variety (BYVMD highly susceptible) with 4.27 g^{-1} min^{-1} at 105 days after sowing. Armugam and Muthukrishnan (1977) conducted an experiment in which they accessed the phenolics and flavonoids contents of two okra cultivars that were susceptible to YVMV. It was observed that the resistant parents exhibited high levels of phenolics and flavonoids, while the susceptible cultivars displayed very low levels of these compounds.

The peroxidase activity was found to increase on inoculation with the virus. On inoculation with the virus the PO activity increased in both resistant and susceptible variety. At 90 DAS and 45 DAG there was significant increase in the enzyme activity and thereafter it was found decreasing. It is clear that the peroxidase activity varies depending on the growth stages of plant. Early virus infection causes more loss as the defense mechanisms are weak and the plant cannot withstand stress. Similar findings were observed in banana bunchy top disease resulting in a notable increase in peroxidase activity within the banana cultivar during infection. Healthy plants were with significantly lower concentration of peroxidase activity compared to *Banana Bunchytop Virus i*nfected plants. *Tobacco mosaic virus* (TMV) infected tobacco plants also showed the same trend (Lagrimini and Rothstein, 1987).

The PO activity in virus inoculated Vimukta variety is even higher than the virus inoculated other varieties. PO activity of Arka Anamika is higher than highly susceptible Anakomban but less than Phule Vimukta and Anjitha. Prakasha (2009)

reported that the phenol activity in Arka Anamika (YVMV resistant variety) was more than that of Puasa Sawani and Hybrid 10 (susceptible varieties).

Polyphenol oxidase is an enzyme responsible for catalyzing the oxidation of phenolic compounds. Phenolic compounds can have antiviral properties and are part of the plant's defense mechanisms against viral infections. PPO activity can lead to the formation of toxic compounds that may restrict the replication or spread of viruses.

Highest PPO activity was observed in BYVMD moderately resistant Phule Vimukta Variety followed by Anjitha and Arka Nikitha. Lowest PPO activity was recorded in the highly susceptible Anakomban variety. PPO activity of Phule Vimukta and Anjitha was highest at 30 DAS 3.27 g^{-1} min^{-1} and 2.47 g^{-1} min^{-1} respectively and was found decreasing after 30 DAS. On analysis of PPO activity after graft inoculation with virus in different varieties at different days after inoculation, no significant difference was observed between the inoculated and uninoculated varieties. PPO activity varied between 1.00 to 3.27g^{-1} min^{-1}. But there was a significant difference in PPO activity at different days after inoculation. PPO activity at 45 DAG was higher than that of 30 DAG and 60 DAG.

The presence of polyphenol oxidases (PPO) enhances plant defense mechanisms. The increase in polyphenol content stimulates phenol production, which effectively limits the spread of the pathogen by generating chlorogenic acid, creating an unfavorable environment for pathogen proliferation (Siddique *et al.*, 2014). Prabu and Warade (2009) made similar observations that wild parents resistant to YVMV exhibited the highest levels of phenolics, peroxidase, polyphenol oxidase activity, and seed soluble protein content, while cultivated okra had the lowest levels of these compounds. Interspecific hybrids displayed intermediate levels between their parents.

PPO activity of five varieties analysed in 5 different growth stages (30,45,75,90 and 105 DAS) ranged between 0.47 to 3.27 g^{-1} min^{-1}. Highest PPO activity was in Phule Vimukta Variety followed by Anjitha and Arka Nikitha.

On analysis of PPO activity after graft inoculation with virus in different varieties at different days after inoculation, no significant difference was observed between the inoculated and uninoculated varieties. PPO activity varied between 1.00 to 3.27g^{-1} min^{-1}. But there was a significant difference in PPO activity at different days after inoculation. PPO activity at 45 DAG was higher than that of 30 DAG and 60 DAG. PPO activity of five varieties analysed in 5 different growth stages (30,45,75,90 and 105 DAS) ranged between 0.47 to 3.27 g^{-1} min^{-1}. Seth *et al.,* (2017) observed defense enzymes like peroxidase and polyphenol oxidase is negatively correlated with the PDI of the YVMV disease even during different growth stages. This indicates the potential of difference in biochemical response as selection indices for screening genotypes resistant to the YVMV disease

PAL is an enzyme involved in the phenylpropanoid pathway, which is responsible for the biosynthesis of various secondary metabolites, including phenolic compounds, flavonoids, and lignin. These secondary metabolites play a role in plant defense against pathogens, including viruses. They can act as antimicrobial compounds and reinforce the plant's cell walls.

PAL activity of infected samples was higher than the PAL activity of healthy samples analysed at 30 DAG. Whereas PAL activity of infected sample was lesser than that of healthy at 45 DAG and 60 DAG. The PAL activity of BYVMV tolerant variety, Phule Vimukta was found higher than that of susceptible variety Anakomban in both uninoculated (161.53 % increase over healthy) and inoculated conditions (290.48% increase over healthy) at 30 DAG. PAL activity varied between 1.30 to 8.57 µg of cinnamic acid g^{-1} fresh weight. A progressive increase in defense related enzymes like peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL) activity is reported in inoculated and susceptible varieties of cowpea (Umamaheswaran, 1996).

PAL activity of five varieties analysed in 5 different growth stages (30,45,75,90 and 105 DAS) ranged between 1.03 to 22 µg of cinnamic acid g^{-1} fresh

weight. Highest PAL activity was in Phule Vimukta variety followed by Anjitha and Arka Nikitha. Lowest PAL activity was recorded in Anakomban variety 1.3 µg of cinnamic acid g^{-1} fresh weight at 30 DAG. PAL activity in different time intervals varied significantly. Highest PAL activity was recorded on 90 DAS followed by 105 DAS. Lowest PAL activity was in early growth stages that is, at 30,45,75 DAS. PAL activity showed a decreasing trend after 90 DAS.

Phenylalanine ammonia lyase, polyphenol oxidase, and peroxidase activities contributed to the development of resistance. Higher defence enzyme concentration may be one of the reasons for disease tolerance in tolerant variety. Thus, ability to produce a higher quantity of defence enzymes can be selected as a parameter for breeding disease resistant plants.

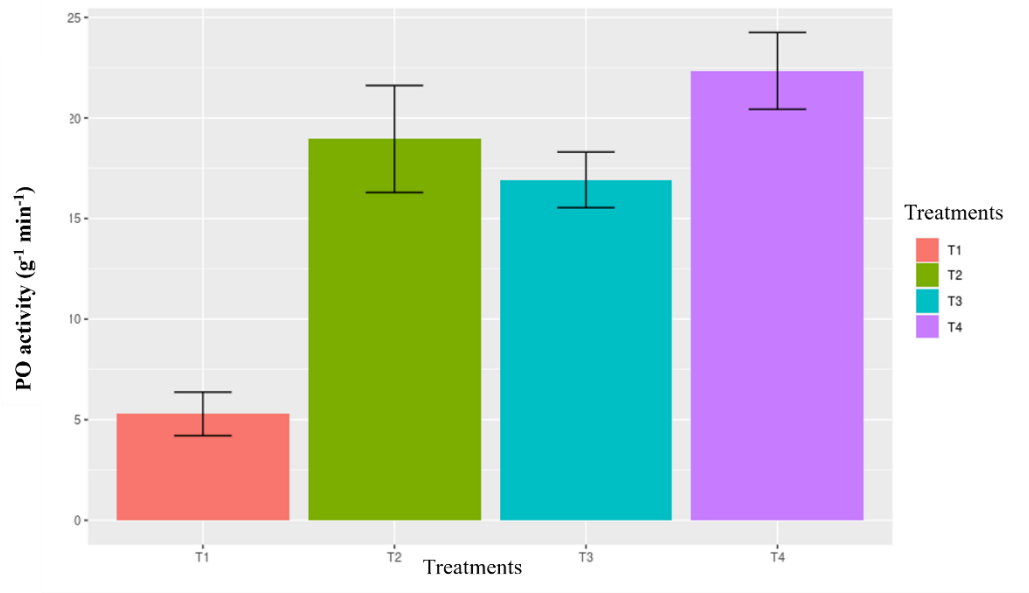

Fig 1. Change in peroxidase activity in okra leaves of susceptible and tolerant variety. T1-Anakomban variety without graft inoculation; T2-Phule Vimukta variety without graft inoculation; T3- Anakomban variety with graft inoculation; T4- Phule Vimukta variety with graft inoculation.

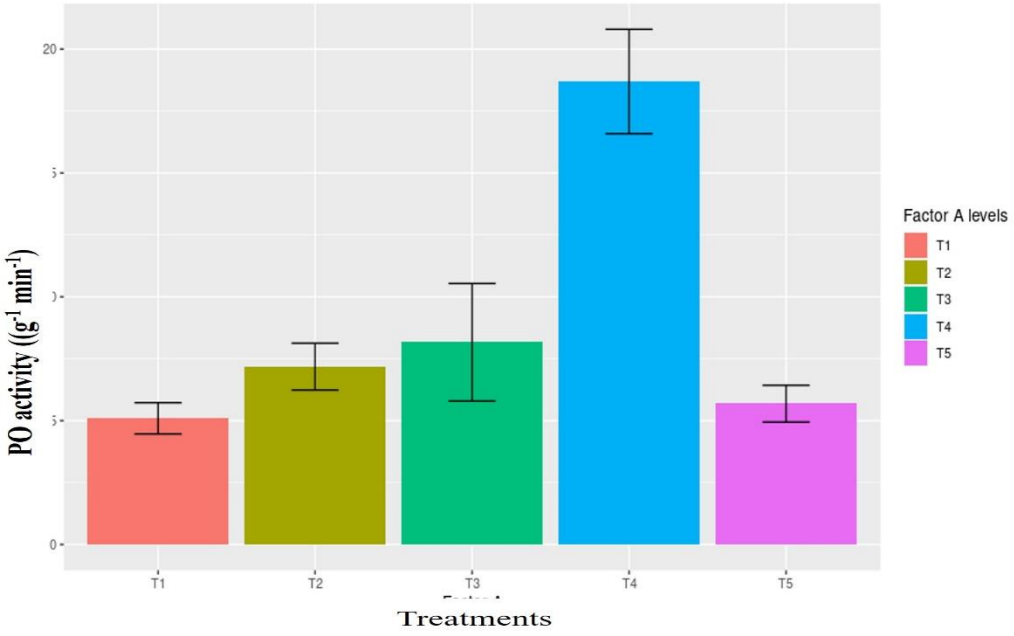

Fig 2. Change in peroxidase activity of different varieties in different growth stages. T1- Anakomban; T2- Arka Anamika; T3- Anjitha; T4- Phule Vimukta; T5- Arka Nikitha.

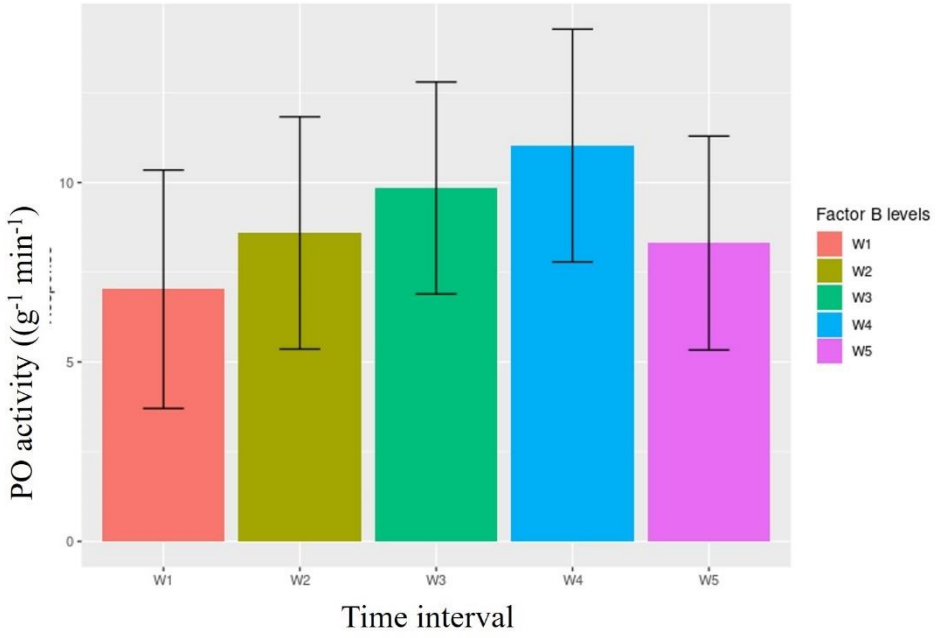

Fig 3. Change in peroxidase activity of different varieties in different growth stages. W1- 30 Days after sowing; W2- 45 Days after sowing; W3- 60 Days after sowing; W4- 90 Days after sowing; W5-105 Days after sowing

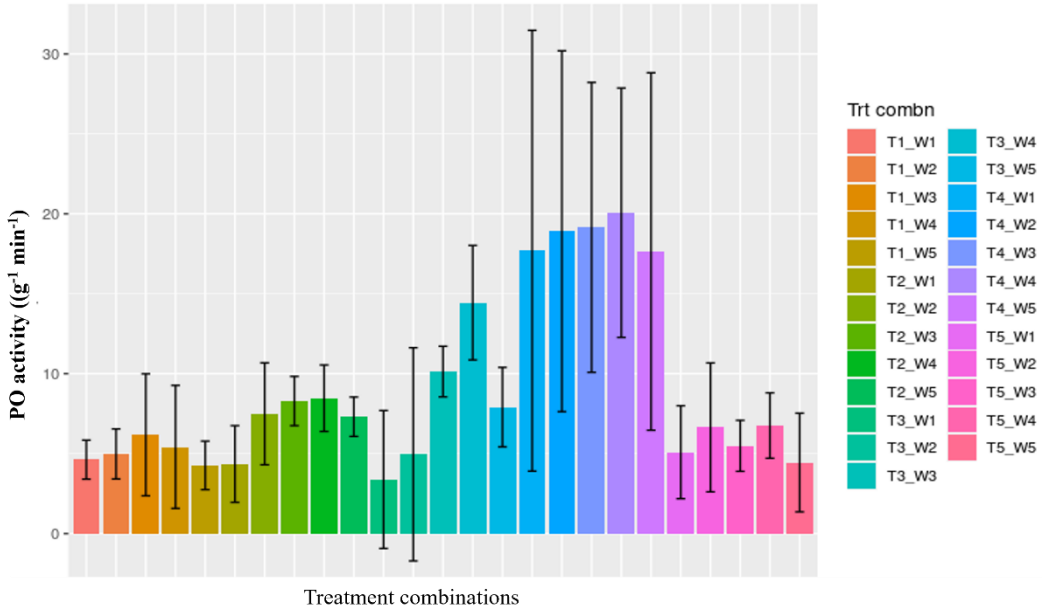

Fig 4. Change in peroxidase activity of different varieties in different growth stages (interaction)

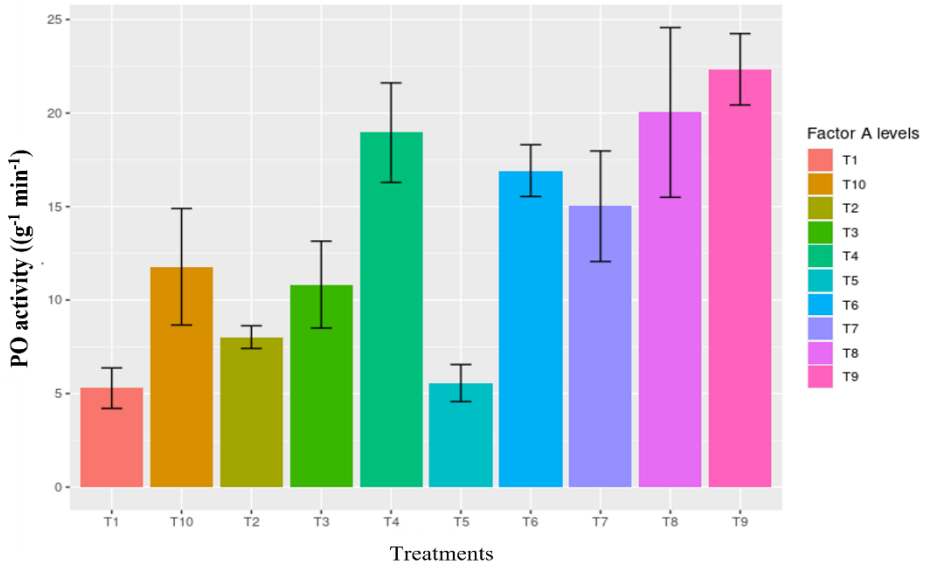

Fig 5. Change in peroxidase activity of different varieties on graft inoculation with virus at different days interval. T1- Anakomban uninoculated; T2- Arka Anamika uninoculated; T3- Anjitha uninoculated T4- Phule Vimukta uninoculated; T5- Arka Nikitha uninoculated; T6 -Virus inoculated Anakomban; T7- Virus inoculated Arka Anamika; T8- Virus inoculated Anjitha; T9- Virus inoculated Phule Vimukta; T10- Virus inoculated Arka Nikitha.

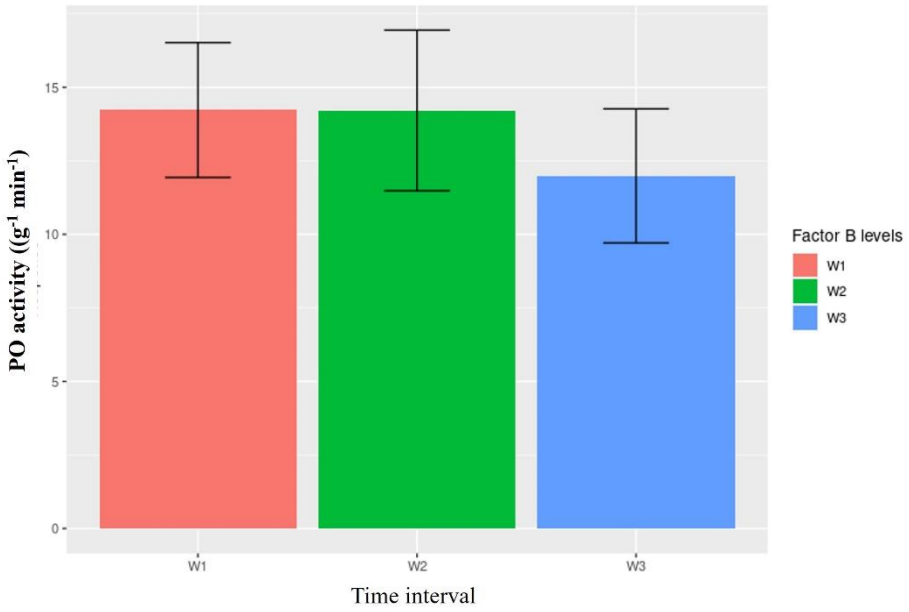

Fig 6. Change in peroxidase activity of different varieties on graft inoculation with virus at different days interval. W1- 45 Days after graft inoculation of T6-T10; W2- 60 Days after graft inoculation of T6-T10; W3- 60 Days after graft inoculation of T6-T10.

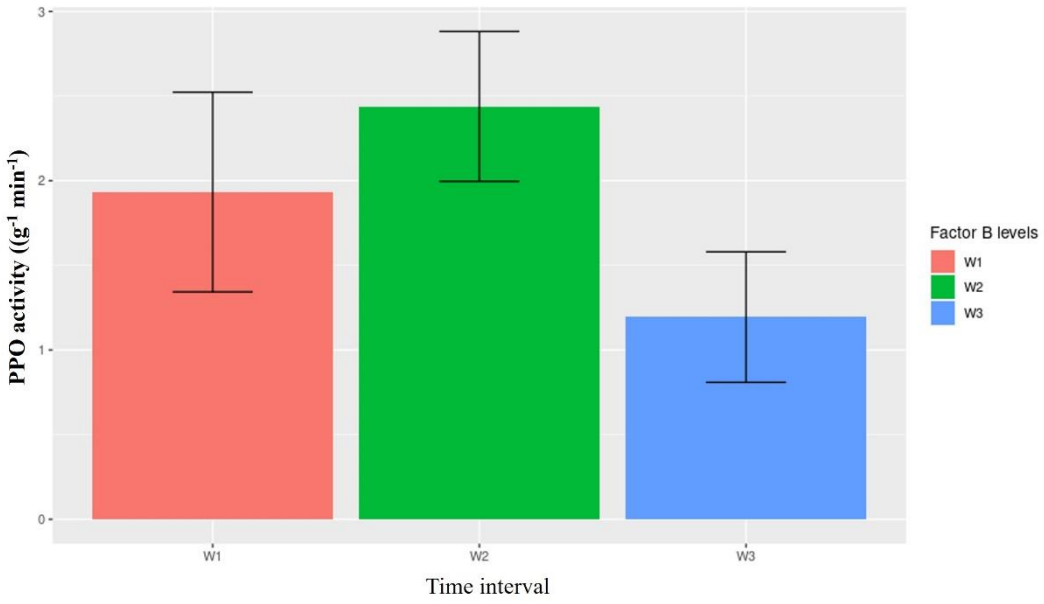

Fig 7. Change in PPO activity in okra leaves of susceptible and tolerant variety. W1- 45 Days after graft inoculation of T3 and T4; W2- 60 Days after graft inoculation of T3 and T4; W3- 60 Days after graft inoculation of T3 and T4.

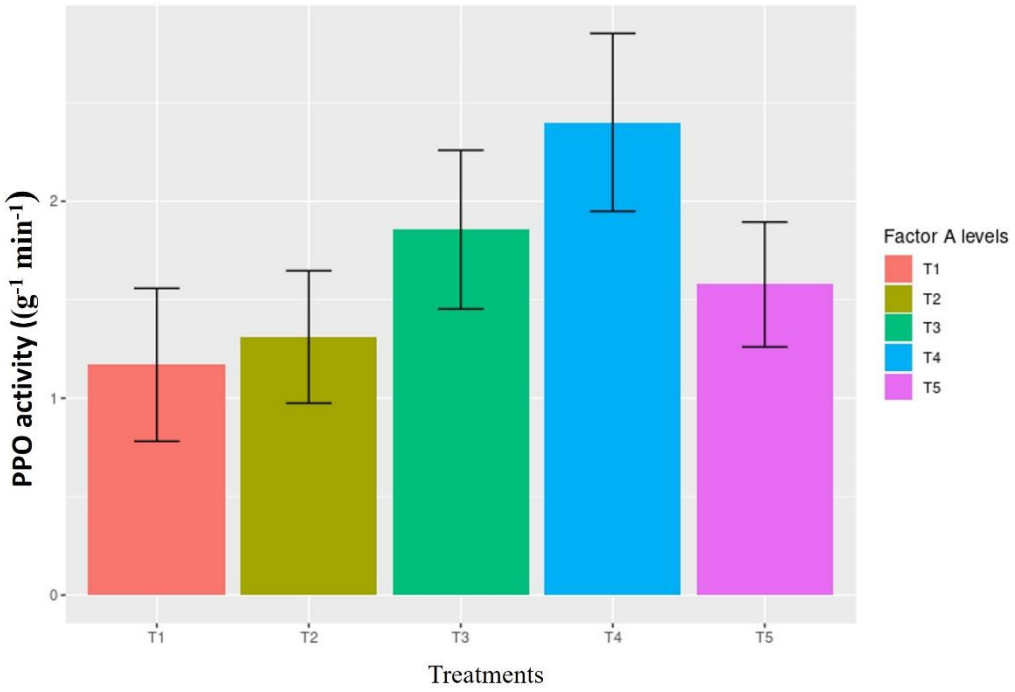

Fig 8. Change in PPO activity of different varieties in different growth stages. T1- Anakomban; T2- Arka Anamika; T3- Anjitha; T4- Phule Vimukta; T5- Arka Nikitha.

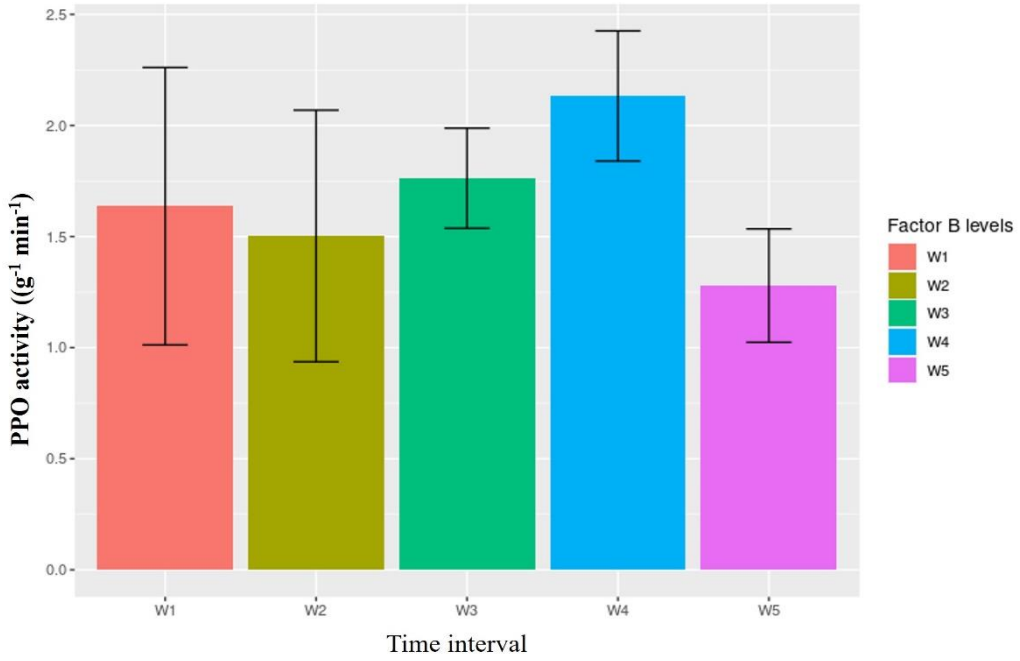

Fig 9. Change in PPO activity of different varieties in different growth stages. W1- 30 Days after sowing; W2- 45 Days after sowing; W3- 60 Days after sowing; W4- 90 Days after sowing; W5-105 Days after sowing.

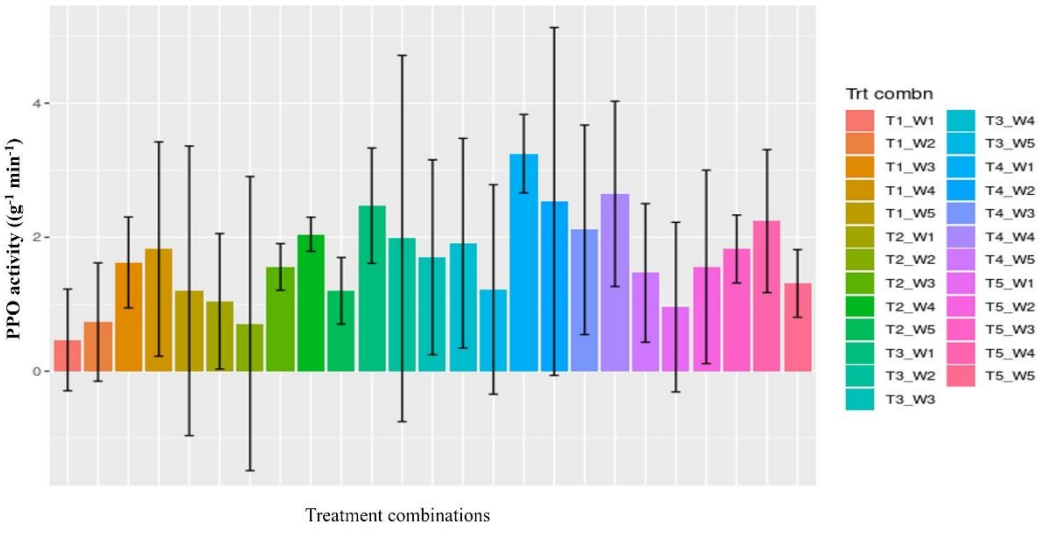

Fig 10. Change in PPO activity of okra leaves in different varieties on different growth stages (interaction)

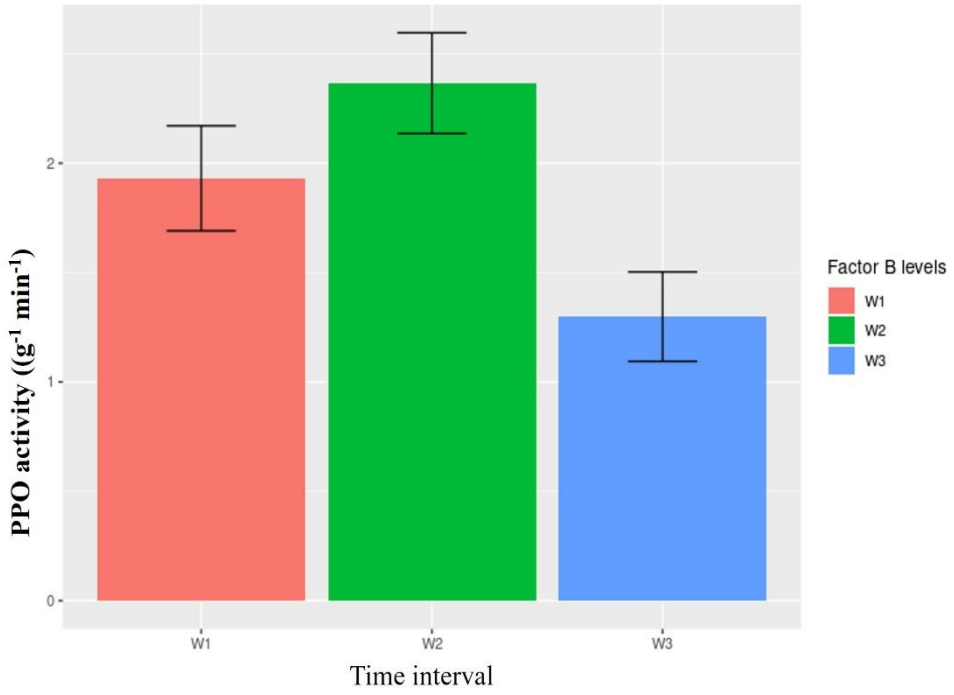

Fig 11. Change in PPO activity of different varieties on graft inoculation with virus at different days interval. W1- 45 Days after graft inoculation of T6-T10; W2- 60 Days after graft inoculation of T6-T10; W3- 60 Days after graft inoculation of T6-T10.

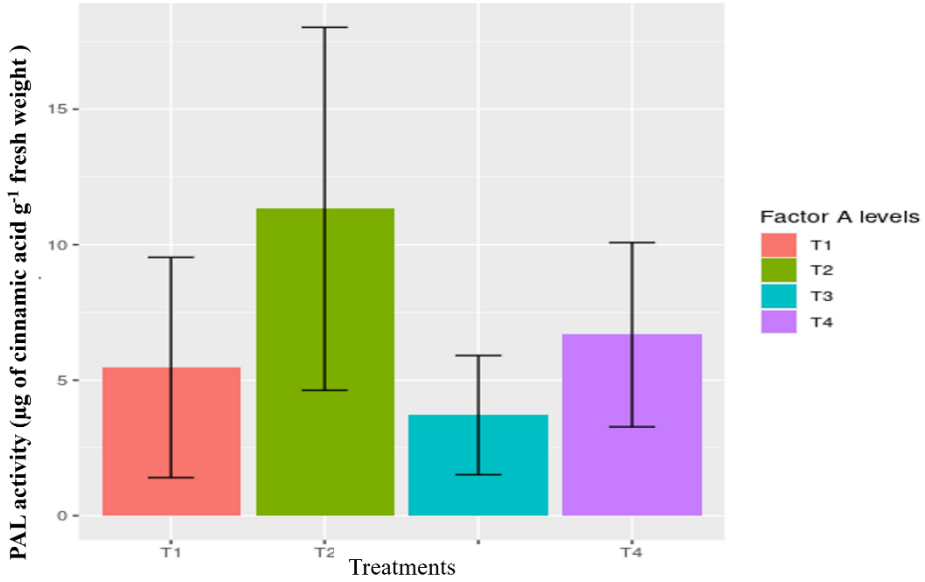

Fig 12. Change in PAL activity in okra leaves of susceptible and tolerant variety. T1-Anakomban variety without graft inoculation; T2- Phule Vimukta variety without graft inoculation; T3- Anakomban variety with graft inoculation; T4- Phule Vimukta variety with graft inoculation.

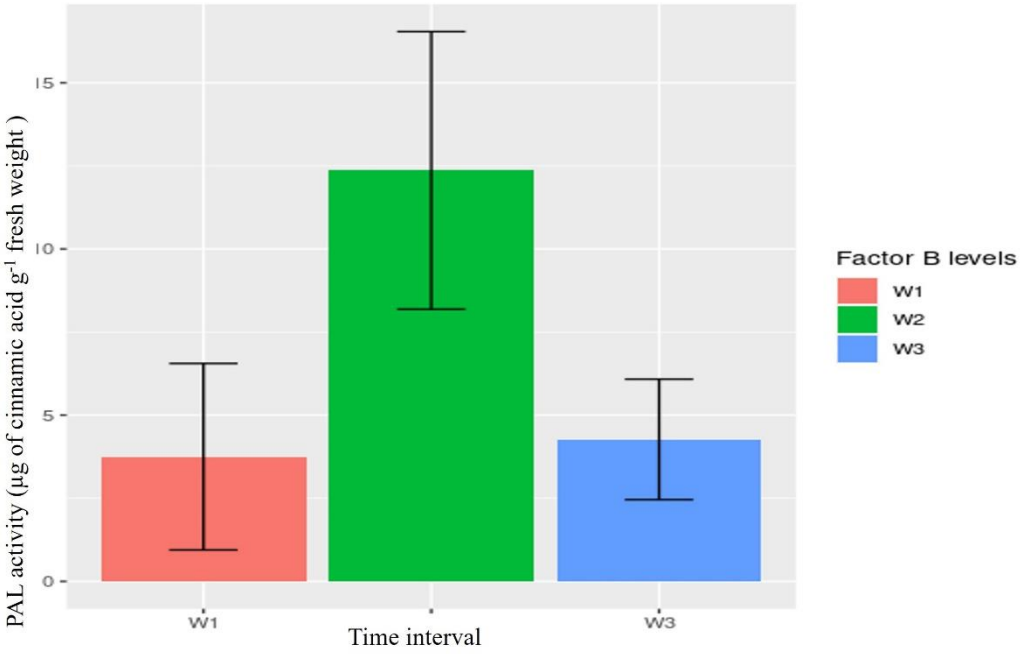

Fig 13. Change in PAL activity in okra leaves of susceptible and tolerant variety. W1- 45 Days after graft inoculation of T3 and T4; W2- 60 Days after graft inoculation of T3 and T4; W3- 60 Days after graft inoculation of T3 and T4.

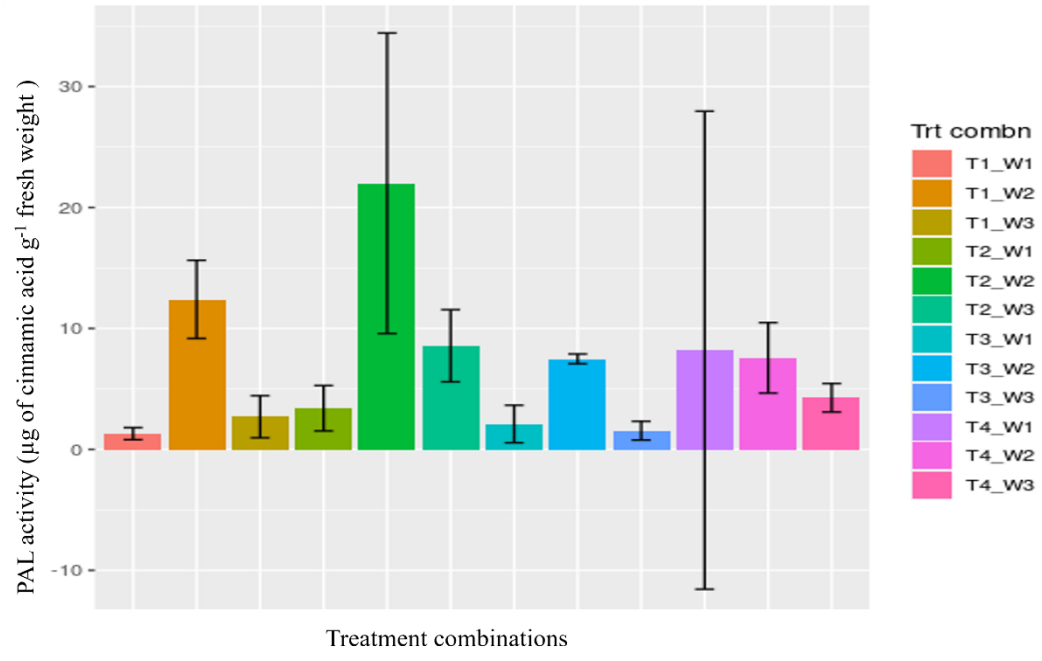

Fig 14. Change in PAL activity in okra leaves of susceptible and tolerant variety(interaction)

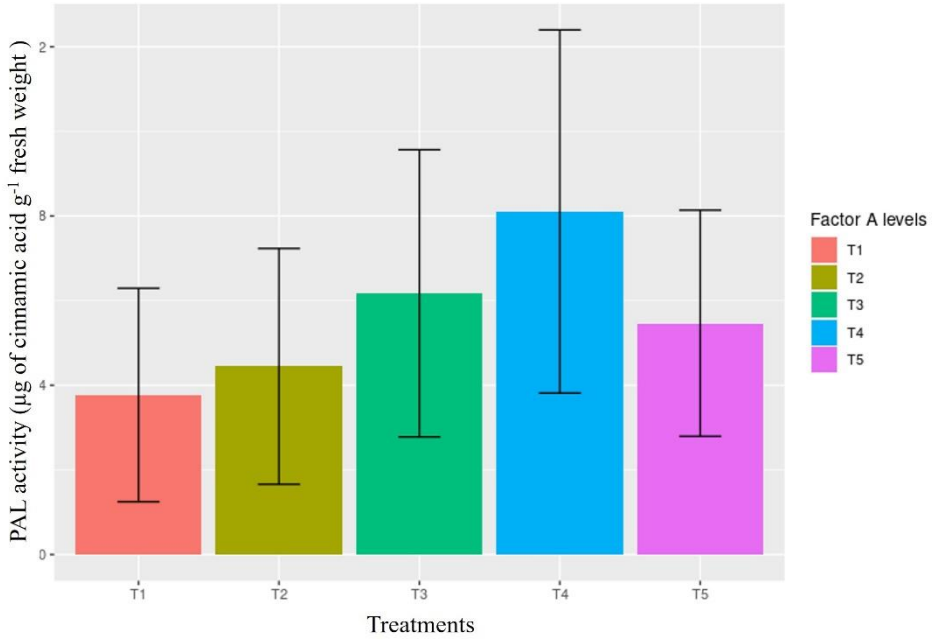

Fig 15. Change in PAL activity in okra leaves of different varieties in different growth stages. T1-Anakomban; T2-Arka Anamika; T3- Anjitha; T4- Phule Vimukta T5-Arka Nikitha

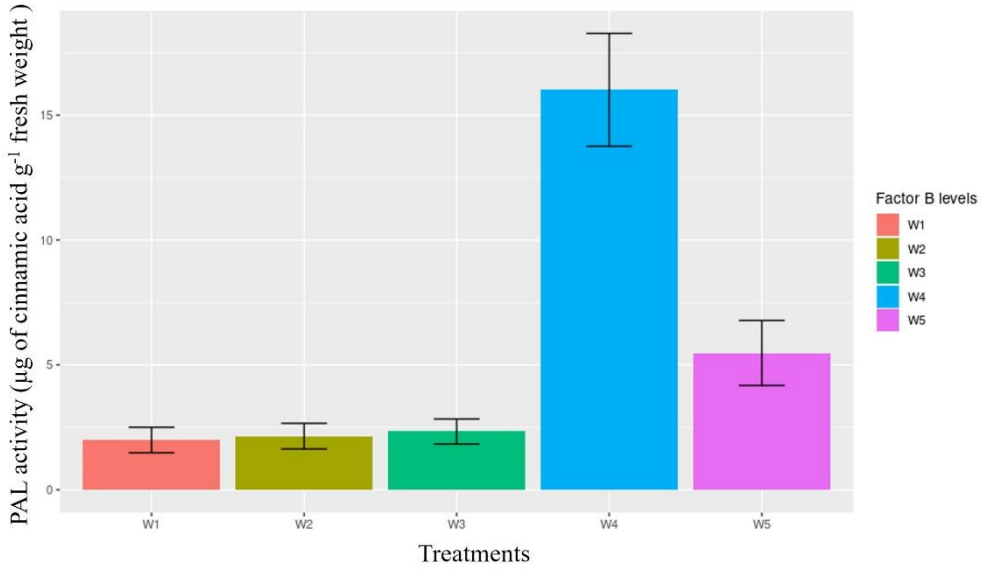

Fig 16. Change in PAL activity in okra leaves of different varieties in different growth stages. W1- 30 Days after sowing; W2- 45 Days after sowing; W3- 60 Days after sowing; W4- 90 Days after sowing; W5-105 Days after sowing.

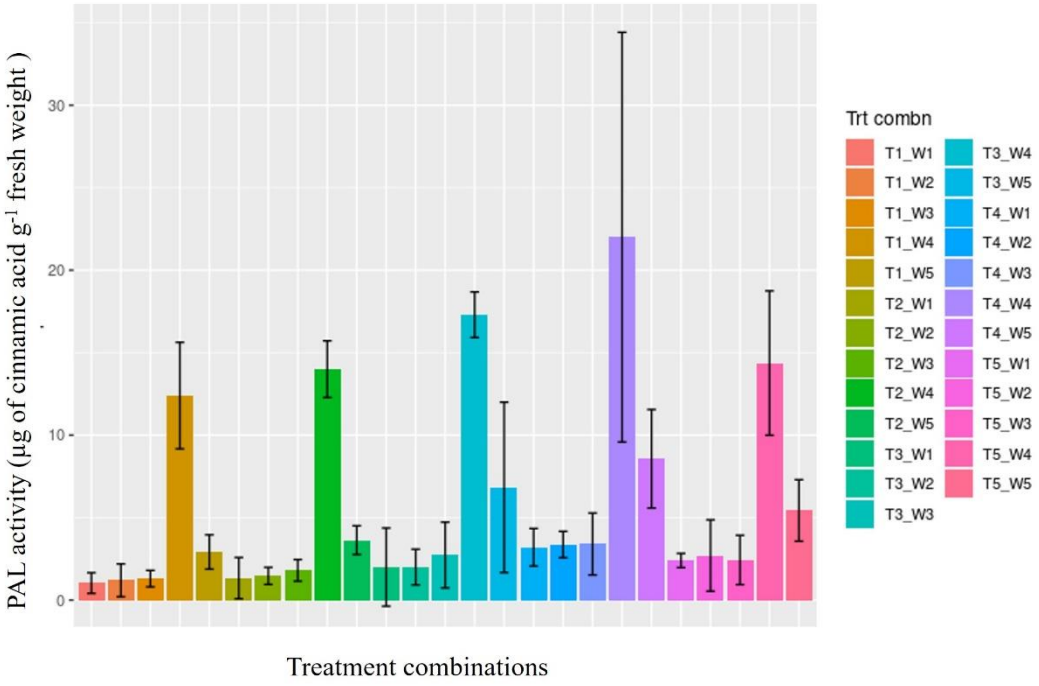

Fig 17. Change in PAL activity of okra leaves in different varieties on different growth stages (interaction)

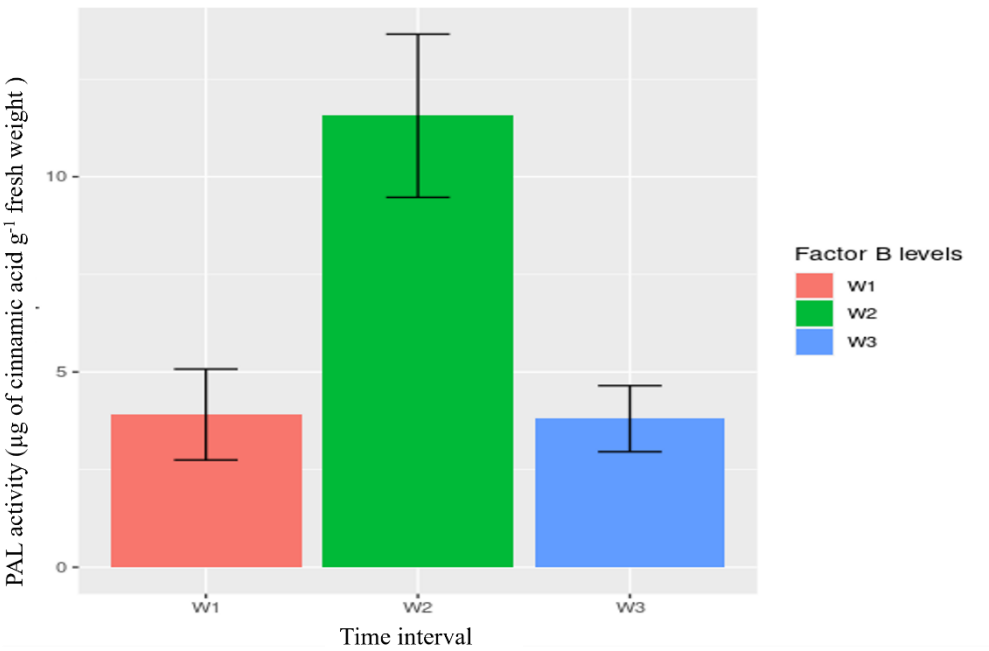

Fig 18. Change in PAL activity of different varieties on graft inoculation with virus at different days interval. W1- 45 Days after graft inoculation of T6-T10; W2- 60 Days after graft inoculation of T6-T10; W3- 60 Days after graft inoculation of T6-T10.

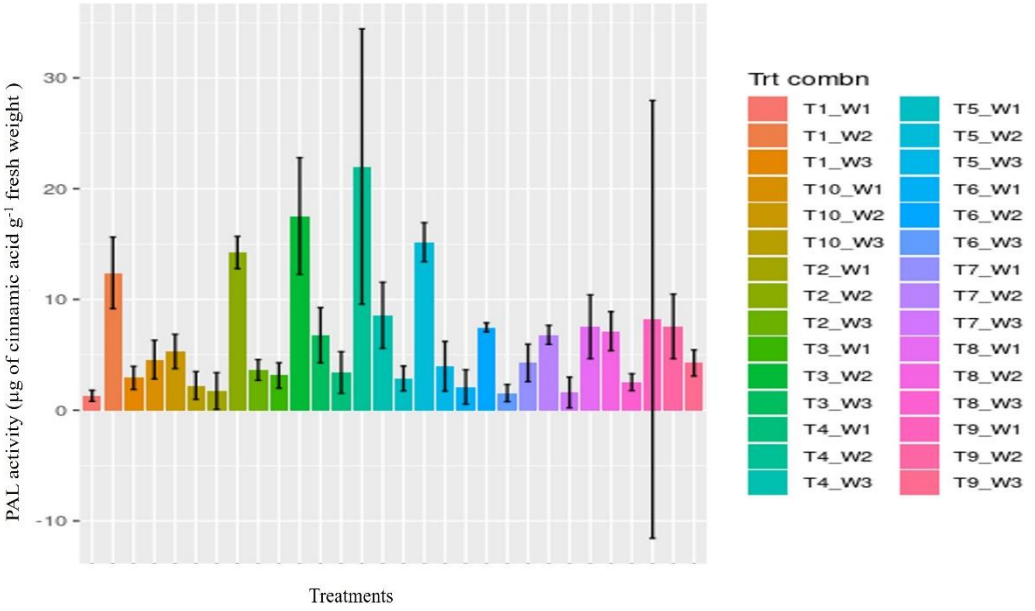

Fig 19. Change in PAL activity in okra leaves of different varieties in different growth stages (interaction)

5.6 MERISTEM CULTURE FOR THE PRODUCTION OF VIRUS-FREE PLANTS

Okra *in-vitro* plant regeneration is very important for genetic transformation as well as production of virus free plants through meristem culture. Virus free plants were regenerated from infected plant's seeds and shoots. Anjitha variety was used for standardization of meristem culture and production of disease-free plants. Bacterial and fungal contamination was a major challenge and different concentrations of sterilization agents were tried. Treating explant with 0.2% mercuric chloride for 1 minute was found to be more effective than other concentrations.

MS media supplemented with 0.5µM benzyl aminopurine + 0.1 µM Naphthalene acetic acid + 0.1µM Gibberellic acid was more effective for meristem establishment with 100 % regeneration of meristem. Nair *et al.,* 1979 also reported this media as the best media for cassava shoot apical meristem *in vitro* propagation. Separation of meristem from explant and growing it in aseptic condition was challenging due to very small size of meristem and chance of getting contaminated during outside exposure. Surface sterilization of meristem leads to loss of meristem and hence proceeded without surface sterilization.

Multiple shoots were initiated in all the meristems kept in MS media with 1.0 mg/l BAP + 0.5 mg/l GA_3. Among the different combinations of BAP, GA_3 and NAA tried, 1.0 mg/l BAP + 0.5 mg/l GA_3 as the suitable media for shoot initiation (Anisuzzaman *et al.* 2010). During shoot multiplication no root induction was noticed in microshoots. MS media with 1 mg/l BAP + 1 mg/l NAA and 0.04 mg/l BAP TDZ was reported as the best media for shoot proliferation in okra by Mallela *et al.* (2009).

Anisuzzaman *et al.* (2010) reported MS media with 1.0 mg/l IBA as the best media for root induction in okra meristem culture. But on the contrary, no root induction was obtained in MS media with 1.0 mg/l IBA. But roots were initiated in MS media with 0.1 mg/l BAP + 0.02 mg/l GA_3 in the present investigation. The

percentage of root initiation is less. BYVMV infected plant's seeds and shoots were taken as explant and cultured aseptically *in vitro.* The regeneration percentage of shoot was higher than seeds. Primary establishment of meristem was done in hormonal media and later transferred into shoot initiation media where 4-6 shoots/ meristem was initiated. The absence of virus in meristem cultured plants was proved by PCR. Anisuzzaman *et al.* (2008) described protocol for mass *in vitro* propagation of okra using meristem culture. Three-week old shoot tip Meristems were isolated from in vitro grown seedlings for production of virus free plants for seed production. MS liquid media with 1.0 mg/l of BAP was efficient for growth of isolated meristems. Semisolid MS medium with 1.0 mg/l of BAP was discovered to be the most efficient for shoot development from meristems.

Based on this research, it can be concluded that the okra yellow vein mosaic disease which exhibits symptoms such as vein clearing and enation in Vellayani area (Thiruvananthapuram, Kerala) is bhendi yellow vein mosaic virus. The presence of the virus was detected in 20% of the seeds, however, seed transmission was not observed during the grow out test. Resistance breakdown was observed in previously identified resistant varieties, namely Varsha Uphar and Arka Anamika. The variety Phule Vimukta, which possesses disease resistance and displays high defense related enzyme activity, can be utilised for breeding purposes in the development of disease-resistant varieties. The meristem culture can be successfully utilized for the production of planting materials free from the virus.

Summary

6. SUMMARY

The study entitled 'Biochemical response of okra to *Bhendi yellow vein mosaic virus* and production of virus free plants' was undertaken at the College of Agriculture, Vellayani, Thiruvananthapuram, Kerala during the years 2021 to 2023 with the objective of studying host pathogen interaction of *bhendi yellow vein mosaic virus* (BYVMV) infecting okra, screening of different genotypes of okra cultivars for disease resistance and production of virus free plants using meristem culture.

Purposive sampling surveys in various locations revealed that, BYVMD is a major constraint in okra cultivation as all plots of surveyed locations were severely infected with okra yellow vein mosaic disease. Purposive sampling survey was done and samples were collected from Vellayani (Thiruvananthapuram), Vellanikkara (Thrissur), and Kumbidi (Palakkad) areas of agro ecological units AEU-8 (Southern laterite) and AEU-10 (North central laterites) respectively during the years 2022-2023. The disease incidence of virus infected okra plants in surveyed locations ranged from 37 to 100 %. The vulnerability index of various plants infected with BYVMV varied from 23.40 to 85.20. The highest vulnerability index was observed in the Arka Anamika variety (85.2), followed by Varsha Uphar (80) and Anakomban (83.53). The okra variety, Anjitha, had the lowest disease incidence of 37.20% and vulnerability index of 23.40. Anakomban and Varsha Uphar varieties exhibited 100% incidence of okra yellow vein mosaic disease in AEU 10, while the disease occurrence was relatively lower in AEU 8. The disease was found to be more prevalent during the flowering and fruiting stages of okra plants. Symptomatology of BYMD infected okra plants revealed typical symptoms of vein clearing, vein thickening and distortion of the leaves. In advanced stages, fruits were also found affected and bleached appearance of fruits was observed in the disease infected plants. Plants that were infected at an early stage showed a higher susceptibility measure compared to plants infected at later stages.

The virus causing BYVMD was graft transmissible and was maintained in Anjitha and Anakomban varieties by wedge grafting. About 87.50 per cent graft transmission of the virus was observed. Anjitha and Anakomban varieties showed 83% and 100% graft transmission respectively. Even though graft union was not observed in all plants, there was successful transmission of virus by graft inoculation. Eight to nine days were taken for symptom development after graft inoculation with virus and vein clearing symptom was observed on newly emerged leaves from the root stock.

Serological detection of virus causing BYVMD was done using Double Antibody Sandwich - Enzyme Linked Immuno Sorbent Assay (DAS-ELISA) and Dot Immuno Binding Assay (DIBA). BYVMD infected samples showed 24.29% and 37.47 % increase over healthy samples in ELISA and DIBA respectively. It was found that BYVMV isolate has a close relationship with tomato leaf curl New Delhi virus. The molecular detection of virus was done using PCR and an expected amplicon of size 520 bp was obtained using Deng primer. The BLAST analysis of the sequence of amplicon from okra with vein clearing symptom and enation symptom showed 96.27 % and 96.67 % similarity with DNA-A segment of bhendi yellow vein mosaic virus isolates. Weed species *Ageratum conizoides* collected from BYVMD infected plots of okra with vein clearing symptom, on molecular detection showed 93.99 % similarity to coat protein of french bean leaf curl Madkeri virus (begomovirus).

Seeds were collected from BYVMD infected plants of different varieties. Seed transmission studies was conducted by grow out test, serological and molecular detection of virus on seed and grow out plant leaves. Out of 286 seeds sown, only 57.14 % germination was observed. None of the seedlings established symptoms till the flowering stage. Molecular analysis of random samples taken from asymptomatic leaves of grow out plants also showed negative reaction to the virus. ELISA using ToLCNDV antiserum also showed negative results confirming absence of seed transmission. But, presence of virus was detected molecularly on whole seeds taken randomly from the seeds collected from infected plants. Out of

20 seeds examined, only four seeds showed a positive reaction. The presence of virus was also not detected on any of the seed parts examined. Hence it can be inferred that the virus is present on seed samples but there is no seed transmission. Germination percentage of seeds from infected plants is less (52%) and it might be due to the impact of virus on seed quality. To ensure complete absence of vector, seeds collected from BYVMD infected plants were also grown in tissue culture media. The PCR analysis of the DNA extracted from leaves collected from *in-vitro* grown plants also showed the absence of amplicon.

Screening of fifteen different genotypes of okra was done by graft transmission. On screening, the Phule Vimukta variety was found to be moderately resistant to BYVMD with a vulnerability index of 13.34. Anjitha variety was moderately susceptible to BYVMD with a vulnerability index of 38.30. Arka Anamika, Anakomban, Arka Nikitha and ten NBPGR accessions (IC 052303, IC 00780, IC 588166, IC 002134, IC 006101, IC 002024, IC 043279, IC 093771, IC 093688, IC 045820) studied were found to be highly susceptible to BYVMD. Graft union was obtained in 14 plants out of 45 grafted plants (31.11%). On screening, Bhendi yellow vein mosaic disease resistance breakdown was observed in the Arka Anamika variety.

Biochemical response of different varieties of okra plants at different growth stages as well as on inoculation with the virus revealed that the activities of defense-related enzymes varied with genotypes and also with the growth stages of the crop. Tolerant varieties were found to possess the highest content of defence enzymes compared to susceptible varieties. PO activity of moderately resistant variety Phule Vimukta was observed with 270.29 % and 35.02 % more enzyme activity than the susceptible variety, Anakomban at 90 days after sowing and 45 days after graft inoculation respectively. PO and PPO activity showed an increasing trend on inoculation with virus while PAL showed a decreasing trend. Phule Vimukta was observed with highest PO (18.69 g^{-1} min^{-1}), PPO (2.40 g^{-1} min^{-1}) and PAL (8.11 µg of cinnamic acid g^{-1} fresh weight) activity followed by Anjitha with PO, PPO and PAL activity of 8.16 g^{-1} min^{-1}, 1.86 g^{-1} min^{-1} and 6.17 µg of cinnamic acid g^{-1} fresh

weight) respectively. Difference in biochemical response can be used as selection indices for screening genotypes resistant to the YVMD.

Standardization of production of virus free plants using meristem culture was done using Anjitha variety. Seeds and shoots from infected plants were used as explant. Treating explant with 0.2% mercuric chloride for 1 minute was found to be more effective than other concentrations. Meristem culture can be successfully done using MS media with BAP (0.5µM), NAA (0.1 µM) and GA_3(0.1µM). Among the different combinations of BAP, GA_3 and NAA tried, 1.0 mg/l BAP + 0.5 mg/l GA_3 as the suitable media for shoot initiation. Pre-established meristems produced 5-6 shoots within 30 days in this medium. Roots were initiated in MS media with BAP (0.1 mg/l) and GA_3 (0.02 mg/l) after 60 days incubation. Virus indexing of meristem cultured plants by PCR confirmed the absence of virus in regenerated plants.

Based on the present study, the virus causing BYMD showing symptoms of vein clearing and enation was found to be BYVMV. Presence of virus was detected on seed (20 %) but seed transmission was not observed in the grow out test. Breakdown of resistance was observed in Varsha Uphar and Arka Anamika which were earlier reported as resistant. The variety Phule Vimukta, with disease resistance and high defense related enzyme activity can be used for breeding purposes for the development of disease resistant varieties. Meristem culture can be successfully used for the production of disease-free planting materials and production of quality seeds.

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Appendices

APPENDICES

APPENDIX I

CTAB Extraction Buffer

C-TAB	2%
Tris- HCl (pH 8.0)	100 mM
EDTA	20 mM
NaCl	2M
β-mercaptoethanol	0.2%
PVP	2%

β-mercaptoethanol and PVP freshly added prior to DNA extraction.

APPENDIX II

TE buffer

Tris- HCl (pH 8.0)	10mM
EDTA	1mM

APPENDIX III

TAE Buffer (50x) for I liter solution

Tris base	242g
Glacial acetic acid	57.1 ml
EDTA (pH 8.0)	100ml

Final volume made up to 1000 ml with distilled water and autoclave before use.

APPENDIX IV

Buffers for DAS- ELISA

1. Phosphate buffer saline (PBS – pH 7.4)

Sodium chloride	8.0g
Potassium dihydrogen phosphate	0.2g
Disodium hydrogen phosphate	1.1g
Potassium chloride	0.2g
Sodium azide	0.2g
Water	1000ml

2. Wash buffer (PBS-T)

Add 0.5ml/L of Tween 20 to 500ml of PBS

3. Coating buffer (pH 9.6)

Sodium carbonate	1.59g
Sodium bicarbonate	2.93g
Sodium azide	0.2g
Water	1000ml

4. Antibody diluents buffer (PBS-TPO)/ conjugate buffer

Add 20g Polyvinyl pyrrolidone and 2g ovalbumin to 1L of PBS-T

5. Blocking solution

Add 50g BSA to 1L of PBS-T

6. Substrate solution (pH 9.8)

Diethanolamine	97 ml
Sodium azide	0.2g
Water	800 ml

(Store in amber colour bottle)

7. Enzyme conjugate diluents buffer

Same as PBS-TPO

8. Antigen extraction buffer (pH 8.5)

Sodium sulphite	0.38g
1M tris HCl	2.5 ml

Make upto 50 ml

APPENDIX V

Buffers for DIBA

1. Stock solution buffer (Tris-buffer saline, TBS – pH 7.5)

0.02M Tris	4.84g
0.5M NaCl	58.48g

Adjust the pH to 7.5 with and make up the volume to 2L. Used as wash solution

2. Antigen extraction buffer (TBS-SDM)

Add 11.25g Diethyl dithiocarbamate (DIECA) to 1L TBS

3. Blocking solution (TBS-SDM)

Add 5.0g spray dried milk (SDM) to 100 ml of TBS

4. Antibody and enzyme-conjugate diluent/buffer

Add antibody of required diluents to TBS-SDM

5. Substrate buffer (pH 9.5)

0.1M Tris	12.11g
0.1M NaCl	5.85g
5mM MgCl2.6H2O	1.01g

Adjust the pH to 9.5 with 1N HCl and make up to 1L.

6. Substrate solution

Solution A

Nitro blue tetrazolium (NBT)	75 mg
Dimethyl formamide (DMFA)	1 ml

Solution B

Bromo chloro indolyl phosphate (BCIP)	50 mg
DMFA	1 ml

Store solutions A and B refrigerated in amber colour bottles. Add 44 µl of NBT and

35 µl of BCIP to 10 ml substrate buffer

7. Fixing solution

10 mM Tris 1.21g

1 mM EDTA 0.29g

Adjust the pH to 7.5 with 1N HCl and make up to 1L. All buffers contain 0.02% sodium azide as a preservative.

APPENDIX VI

Buffers for enzyme analysis

1. 0.1M Sodium phosphate buffer (pH – 6.5)

Stock solutions

A: 0.2M Solution of monobasic sodium phosphate (27.8 g in 1000 ml)

B: 0.2M Solution of dibasic sodium phosphate (53.65 g of Na2.HPO4.12 H2O in 1000 ml)

68.5 ml of A mixed with 31.5 ml of B diluted to a total of 200 ml

2. 0.1M Borate buffer (pH – 8.8)

Stock solutions

A: 0.2M Solution of Boric acid (12.4 g in 1000 ml)

B: 0.05M Solution of Borax (19.05 g in 1000 ml)

50 ml of A is mixed with 30 ml of B, diluted to a total of 200 ml

APPENDIX VII

Stock solutions for Murashige and Skoog (MS) Medium

Stock	Chemical	Quantity for 1L of MS (mg)	Concentration of stock	Volume of stock	Quantity for preparing stock (mg^{-1})	Volume required for 1L of medium
Macro elements						
A	NH_4NO_3 KNO3 $MgSO_4.7H_2O$ KH_2PO_4	1650 1900 370 170	40X	100 ml	6600 7600 1480 680	25.0
B	CaCl2.2H2O	440	200X	100 ml	8800	5.0
Micro elements						
C	KI H_3BO_3 $MnSO_4.H_2O$ $ZnSO_4.7H_2O$ $Na2MoO_4.2H_2O$	0.83 6.20 16.9 8.60 0.25	1000X	100 ml	83 620 1690 860 25	1.0
D	**Iron source** $FeSO_4.7H_2O$ $Na_2EDTA.2H_2O$	 27.8 37.3	200X	100 ml	 556.0 746.0	5.0

E	$CuSO_4.5H_2O$ $CoCl_2.6H_2O$	0.025 0.025	5000X	100 ml	12.5 12.5	0.2
Organic supplements						
F	Vitamins Nicotinic acid Pyridoxine-HCl Thiamine-HCl Glycine	 0.5 0.5 0.1 2.0	1000X	100 ml	 50.0 50.0 10.0 200.0	1.0
Components directly added.						
	Myoinositol	100 mg/L				
Carbon source						
	Sucrose	30 g/L				
Solidifying Agent						
	Agar	6 g/L				

BIOCHEMICAL RESPONSE OF OKRA TO *Bhendi yellow vein mosaic virus* AND PRODUCTION OF VIRUS FREE PLANTS

By

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Abstract of the thesis

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ABSTRACT

The research entitled 'Biochemical response of okra to *Bhendi Yellow Vein Mosaic Virus* and production of virus free plants' was undertaken at the College of Agriculture, Vellayani, Thiruvananthapuram during the years 2021 to 2023 with the objective of host pathogen interaction of *Bhendi yellow vein mosaic virus* (BYVMV) infecting okra, screening of different genotypes of okra cultivars for disease resistance and production of virus free plants using meristem culture.

Purposive sampling survey was done and samples were collected from AEU 8 and AEU 10. The disease incidence of virus infected okra plants in surveyed locations ranged from 37 to 100 %. And vulnerability index varied from 23.40 to 85.20. Highest V. I. was observed in Arka Anamika (85.2) followed by varieties, Varsha Uphar (80) and Anakomban (83.53). The okra variety, Anjitha, recorded lowest disease incidence of 37.20 per cent and vulnerability index of 23.40. Disease incidence was observed to be high at the flowering and fruiting stage.

The virus causing BYVMD was serologically detected using ELISA and DIBA and found that BYVMV isolate have close relationship with tomato leaf curl New Delhi virus. The molecular detection of virus was done using PCR and an expected amplicon of size 520 bp was obtained using Deng primer. The BLAST analysis of the sequence of amplicon from okra with vein clearing symptom and enation symptom showed 96.27 % and 96.67 % similarity with DNA-A segment of bhendi yellow vein mosaic virus isolate.

Out of 286 seeds collected from infected plants seeds sown, only 57.14 % germination was observed. None of the seedlings established symptoms till flowering stage. Molecular analysis of random samples taken from asymptomatic leaves of grow out plants also showed negative reaction to the virus. ELISA also showed negative results confirming absence of seed transmission. But, presence of virus was detected molecularly on whole seeds taken randomly from the seeds collected from infected plants. Out of 20 seeds examined only four seeds showed positive reaction. The presence of virus was also not detected on any of the seed

parts examined. Hence it can be inferred that the virus is present on seed samples but there is no seed transmission.

On screening of 15varieties, Phule Vimukta variety (V. I.-13.34) was found to be moderately resistant to BYVMD and Anjitha variety (V. I.- 38.30) was moderately susceptible to BYVMD. Arka Anamika, Anakomban, Arka Nikitha and ten NBPGR accessions studied were found to be highly susceptible to BYVMD. (IC 052303, IC 00780, IC 588166, IC 002134, IC 006101, IC 002024, IC 043279, IC 093771, IC 093688, IC 045820)

Defense-related enzymes activities varied with genotypes and also with the growth stages of the crop. Tolerant varieties were found to possess the highest content of defence enzymes compared to susceptible varieties. PO activity of moderately resistant variety Phule Vimukta was observed with 270.29 % and 35.02 % more enzyme activity than the susceptible variety, Anakomban at 90 days after sowing and 45 days after graft inoculation respectively. PO and PPO activity showed an increasing trend on inoculation with virus while PAL showed a decreasing trend.

Standardisation of production of virus free plants using meristem culture was done. Meristem culture can be successfully done using MS media with BAP (0.5µM), NAA (0.1 µM) and GA_3 (0.1µM). Virus indexing of meristem cultured plants by PCR confirmed the absence of virus in regenerated plants.

Based on the present study, the virus causing BYMD showing symptoms of vein clearing and enation was found to be BYVMV. Presence of virus was detected on seed (20 %) but seed transmission was not observed in grow out test. Breakdown of resistance was observed in Varsha Uphar and Arka Anamika which were earlier reported as resistant. The variety Phule Vimukta, with disease resistance and high defense related enzyme activity can be used for breeding purposes for the development of disease resistant varieties. Meristem culture can be successfully used for the production of disease-free planting materials and production of quality seeds.

സംഗ്രഹം

'ബെണ്ടി യെല്ലോ വെയ്ൻ മൊസൈക് വൈറസിനോടുള്ള വെണ്ടയുടെ ബയോകെമിക്കൽ പ്രതികരണവും വൈറസ് രഹിത സസ്യങ്ങളുടെ ഉൽപാദനവും' എന്ന തലക്കെട്ടിലുള്ള ഗവേഷണം, 2021- 2023 കാലയളവിൽ തിരുവനന്തപുരം വെള്ളയാണി കാർഷിക കോളേജിൽ നടത്തി. രോഗ പ്രതിരോധ ശേഷി ഉള്ള ഇനങ്ങളെ കണ്ടെത്തുക, വൈറസിനെതിരെയുള്ള വെണ്ടയിലെ രാസപ്രവത്തനങ്ങളിലെ വ്യതിയാനം കണ്ടെത്തുക, വിത്തു വഴിയുള്ള വൈറസിന്റെ വ്യാപനം പഠിക്കുക, മെറിസ്റ്റം കൾച്ചർ ഉപയോഗിച്ച് വൈറസ് രഹിത സസ്യങ്ങളുടെ ഉത്പാദനം എന്നിവ ലക്ഷ്യമിട്ടാണ് ഈ പഠനം നടത്തിയത്.

തിരുവനന്തപുരം, തൃശൂർ, പാലക്കാട് പ്രദേശങ്ങളിൽ സർവേ നടത്തുകയും സാമ്പിളുകൾ ശേഖരിക്കുകയും ചെയ്തു. സർവേ നടത്തിയ സ്ഥലങ്ങളിലെ വെണ്ടച്ചെടികളിൽ വൈറസ് രോഗവ്യാപനം 37 മുതൽ 100% വരെയും,. വൾനറബിലിറ്റി ഇൻഡക്സ് 23.40 മുതൽ 85.20 വരെയും കാണപ്പെട്ടു. ഏറ്റവും ഉയർന്ന വൾനറബിലിറ്റി ഇൻഡക്സ് അർക്ക അനാമിക (85.2), വർഷ ഉപഹാർ (80), ആനക്കൊമ്പൻ ഇനങ്ങളിൽ രേഖപ്പെടുത്തി (83.53). ഏറ്റവും കുറഞ്ഞ രോഗബാധയായ 37.20 ശതമാനവും വൾനറബിലിറ്റി ഇൻഡക്സ് 23.40 വും രേഖപ്പെടുത്തിയത് അഞ്ജിത ഇനത്തിലാണ്. എ.ഇ.യു 10 ൽ100 ശതമാനം ഒക്ര യെല്ലോ വെയിൻ മൊസൈക് രോഗം കാണപ്പെട്ടപ്പോൾ, എ.ഇ.യു 8 ൽ രോഗബാധ താരതമ്യേന കുറവായിരുന്നു. രോഗബാധ കൂടുതലായി കാണപ്പെടുന്നത് വെണ്ട ചെടികൾ പൂവിടുകയും കായ്ക്കുകയും ചെയ്യുന്ന ഘട്ടത്തിലാണ്. ഗ്രാഫ്റ്റിങ് (ഒട്ടിക്കൽ) വഴി പടരുന്ന വൈറസ്, അഞ്ജിത, ആനക്കൊമ്പൻ ഇനങ്ങളിൽ വെഡ്ജ് ഗ്രാഫ്റ്റിംഗ് വഴി പരിപാലിക്കപ്പെട്ടു. ഗ്രാഫ്റ്റിംഗിലൂടെ 87.50 ശതമാനം വൈറസ് പകരുന്നതായി കണ്ടെത്തി. അഞ്ജിത, ആനക്കൊമ്പൻ ഇനങ്ങളിൽ യഥാക്രമം 83%, 100% ഗ്രാഫ്റ്റ് ട്രാൻസ്മിഷൻ കാണിച്ചു. എല്ലാ ചെടികളിലും ഗ്രാഫ്റ്റ് യൂണിയൻ നിരീക്ഷിക്കപ്പെട്ടില്ലെങ്കിലും ഗ്രാഫ്റ്റ് ഇനോക്കുലേഷൻ വഴി വൈറസ് വിജയകരമായി പകരുന്നു.

എലിസ, ഡിബ എന്നിവ ഉപയോഗിച്ച് വെണ്ടയിലെ മൊസൈക് രോഗത്തിന് കാരണമാകുന്ന വൈറസ് സെറോളജിക്കലായി പരിശോധിക്കുകയും അതിന് ടൊമാറ്റോ ലീഫ് കേൾ ന്യൂഡൽഹി വൈറസുമായി അടുത്ത

ബന്ധമുണ്ടെന്നു കണ്ടെത്തുകയും ചെയ്തു. ഡെങ് പ്രൈമർ ഉപയോഗിച്ച് പിസിആർ ചെയ്തപ്പോൾ 520 ബിപി വലുപ്പമുള്ള ആംപ്ലിക്കൺ ലഭിച്ചു. വെയ്ൻ ക്ലീറിങ് ലക്ഷണവും എനേഷൻ ലക്ഷണവുമുള്ള വെണ്ടയിൽ നിന്നും വേർതിരിച്ചെടുത്ത വൈറസിന്റെ തന്മാത്ര സ്വഭാവം ബെൻഡി യെല്ലോ വെയിൻ മൊസൈക് വൈറസ് ഐസൊലേറ്റിന്റെ ഡിഎൻഎ-എ സെഗ്മെന്റുമായി 96.27 ശതമാനവും 96.67 ശതമാനവും സാമ്യം കാണിച്ചു.

മൊസൈക് രോഗബാധിത സസ്യങ്ങളിൽ നിന്ന് ശേഖരിച്ച വിത്തുകൾ ഉപയോഗിച്ച് വിത്ത് വഴിയുള്ള രോഗ വ്യാപനം പഠിച്ചു. വിതച്ച 286 വിത്തുകളിൽ 57.14 ശതമാനം മാത്രമാണ് മുളച്ചത്. തൈകളൊന്നും രോഗലക്ഷണങ്ങൾ പ്രകടിപ്പിച്ചില്ല. ഈ ചെടികളുടെ ലക്ഷണങ്ങളില്ലാത്ത ഇലകളിൽ നിന്ന് എടുത്ത സാമ്പിളുകളുടെ തന്മാത്രാ വിശകലനവും വൈറസ് ഇല്ല എന്നാണ് കാണിച്ചത്. ടൊമാറ്റോ ലീഫ് കേൾ ന്യൂഡൽഹി വൈറസ് ആന്റിസെറം ഉപയോഗിച്ചുള്ള എലിസയും വൈറസിന്റെ വിത്ത് വഴി ഉള്ള വ്യാപനത്തിന്റെ അഭാവം സ്ഥിരീകരിക്കുന്ന നെഗറ്റീവ് ഫലങ്ങൾ കാണിച്ചു. എന്നാൽ, രോഗബാധിതമായ സസ്യങ്ങളിൽ നിന്ന് ശേഖരിച്ച വിത്തുകളിൽ വൈറസിന്റെ സാന്നിധ്യം പി സി ആർ ൽ കണ്ടെത്തി. പരിശോധിച്ച 20 വിത്തുകളിൽ നാല് വിത്തുകൾ മാത്രമാണ് പോസിറ്റീവ് പ്രതികരണം കാണിച്ചത്. എന്നാൽ വിത്തിന്റെ ഭാഗങ്ങൾ വേർതിരിച്ചു നടത്തിയ പരിശോധനയിൽ വൈറസ് ഏതു ഭാഗത്താണ് കാണപ്പെടുന്നത് എന്ന് സ്ഥിതീകരിക്കാൻ സാധിച്ചില്ല. അതിനാൽ, വിത്ത് സാമ്പിളുകളിൽ വൈറസ് ഉണ്ടെങ്കിലും വിത്തിലൂടെ രോഗം പടരുന്നില്ലെന്ന് അനുമാനിക്കാം.

വെണ്ടയുടെ പതിനഞ്ച് വ്യത്യസ്ത ജീനോടൈപ്പുകളുടെ സ്ക്രീനിംഗ് ഗ്രാഫ്റ്റ് ഇനോക്കുലഷൻ വഴി നടത്തിയതിൽനിന്നും, ഫൂലെ വിമുക്ത, അഞ്ജിത ഇനങ്ങൾ മിതമായി വൈറസിനെ പ്രതിരോധിക്കുമെന്ന് കണ്ടെത്തി. അർക്ക അനാമിക, ആനക്കൊമ്പൻ, അർക്ക നികിത, പത്ത് എൻ. ബി. പി. ജി. ആർ. അക്സെഷൻസ് (ഐസി 052303, ഐസി 00780, ഐസി 588166, ഐസി 002134, ഐസി06101, ഐസി 002024, ഐസി 043279, ഐസി 093771, ഐസി 093688, ഐസി 045820) എന്നിവക്കു ബിവൈവിഎംഡി പ്രതിരോധശേഷി വളരെ കുറവാണെന്നും കണ്ടെത്തി.

വിവിധ ഇനം വെണ്ടചെടികളിൽ വിവിധ വളർച്ചാ ഘട്ടങ്ങളിലും വൈറസ് ബാധിക്കുമ്പോഴുമുള്ള രോഗപ്രതിരോധവുമായി ബന്ധപ്പെട്ട എൻസൈമുകളുടെ പ്രവർത്തനങ്ങൾ പരിശോധിച്ചപ്പോൾ അവ ജീനോടൈപ്പുകൾക്കും വിളയുടെ വളർച്ചാ ഘട്ടങ്ങൾക്കും അനുസരിച്ച് വ്യത്യാസപ്പെട്ടിരിക്കുന്നുവെന്ന് തെളിഞ്ഞു. മിതമായി വൈറസിനെ പ്രതിരോധിക്കുന്ന ഇനങ്ങളിൽ പ്രതിരോധശേഷി കുറഞ്ഞ ഇങ്ങളെക്കാൾ കൂടുതൽ എൻസൈമുകളുടെ പ്രവർത്തനം കാണപ്പെട്ടു മിതമായ പ്രതിരോധശേഷിയുള്ള ഇനമായ ഫുലെ വിമുക്തയുടെ പെറോക്സിഡേസ് പ്രവർത്തനം, വിതച്ച് 90 ദിവസത്തിലും ഗ്രാഫ്റ്റ് ഇനോക്കുലേഷൻ കഴിഞ്ഞ് 45 ദിവസത്തിലും യഥാക്രമം 270.29 ശതമാനവും 35.02 ശതമാനവും കൂടുതലാണ്. പെറോക്സിഡേസ്, പോളിഫിനോൾ ഓക്സിഡേസ് പ്രവർത്തനങ്ങൾ വൈറസ് ഇനോക്കുലേഷനിൽ വർധിക്കുമ്പോൾ, ഫെനിൽ അലാനിൻ അമോണിയലയേസിന്റെ പ്രവർത്തനം കുറഞ്ഞു വരുന്നതായി കാണാം.

മെറിസ്റ്റെം കൾച്ചർ ഉപയോഗിച്ച് വൈറസ് രഹിത സസ്യങ്ങളുടെ ഉൽപ്പാദനം വിജയകരമായി നടത്തി. പി. സി. ആർ. വഴി മെറിസ്റ്റെം കൾച്ചർ ചെയ്ത സസ്യങ്ങളിൽ വൈറസിന്റെ അഭാവം സ്ഥിരീകരിച്ചു.

ഈ പഠനത്തിന്റെ അടിസ്ഥാനത്തിൽ, വിത്തിൽ വൈറസിന്റെ സാന്നിധ്യം കണ്ടെത്തി (20%) എന്നാൽ വിത്തിൽ നിന്നും അടുത്ത തലമുറയിലേക്ക് പകരുന്നതായി നിരീക്ഷിച്ചില്ല. നേരത്തെ പ്രതിരോധശേഷിയുള്ളതായി റിപ്പോർട്ട് ചെയ്യപ്പെട്ട വർഷ ഉപഹാർ, അർക്ക അനാമിക എന്നിവയുടെ പ്രതിരോധശേഷി നഷ്ടപ്പെട്ടതായി നിരീക്ഷിക്കപ്പെട്ടു. രോഗ പ്രതിരോധശേഷിയും ഉയർന്ന പ്രതിരോധവുമായി ബന്ധപ്പെട്ട എൻസൈം പ്രവർത്തനവുമുള്ള ഫുലെ വിമുക്ത ഇനം രോഗ പ്രതിരോധശേഷിയുള്ള ഇനങ്ങൾ വികസിപ്പിക്കുന്നതിനായി ഉപയോഗിക്കാം. രോഗരഹിത തൈകളുടെ ഉൽപാദനത്തിനും ഗുണനിലവാരമുള്ള വിത്തുകളുടെ ഉൽപാദനത്തിനും മെറിസ്റ്റെം കൾച്ചർ വിജയകരമായി ഉപയോഗിക്കാം.