IMMUNOMOLECULAR DETECTION AND CHARACTERISATION OF *POTYVIRUSES* INFECTING COWPEA (*Vigna unguiculata* (L.) Walp.) AND PAPAYA (*Carica papaya* L.)

by

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DECLARATION

I hereby declare that this thesis entitled **"IMMUNOMOLECULAR DETECTION AND CHARACTERISATION OF *POTYVIRUSES* INFECTING COWPEA (*Vigna unguiculata* (L.) Walp.) AND PAPAYA (*Carica papaya* L.)"** 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, associateship, fellowship or other similar title of any University or Society.

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Dedicated to my dearest mother

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LIST OF ABBREVIATIONS

µg	Microgram
µl	Microlitre
ALMV	*Alfalfa mosaic virus*
APS	Ammonium persulphate
AzMV	*Adzuki bean mosaic virus*
BBrMV	*Banana bract mosaic virus*
BCMV	*Bean common mosaic virus*
BgMV	*Bottle gourd mosaic virus*
BGYMV	*Blackgram yellow mosaic virus*
BLAST	Basic Local Alignment Search Tool
BlCMV	*Blackeye cowpea mosaic virus*
bp	Base pair
BSA	Bovine serum albumin
BtMV	*Beet mosaic virus*
BYMV	*Bean yellow mosaic virus*
CABMV	*Cowpea aphid-borne mosaic virus*
ClYVV	*Clover yellow vein virus*
CMV	*Cucumber mosaic cucumovirus*
CP	Coat protein
CPMV	*Cowpea mosaic virus*
CPMMV	*Cowpea mild mottle calarvirus*
DAC-ELISA	Direct antigen coating-Enzyme linked immunosorbent assay
DAI	Days after inoculation
DIBA	Dot immune binding assay
DIECA	Diethyl dithiocarbamate
EMoV	*Egg-plant mottle virus*

EST	Esterase
GOT	Glutamate oxaloacetate transaminase
h	Hour
ha	Hectare
HMV	*Henbane mosaic virus*
IDH	Isocitrate dehydrogenase
kb	Kilobase
kDa	Kilo dalton
kg	Kilo gram
L	Litre
LDMV	*Leaf distortion mosaic virus*
L-DOPA	L-3,4-dihydroxy phenyl alanine
LMV	*Lettuce mosaic virus*
M	Molar
MAb	Monoclonal antibody
MDH	Malate dehydrogenase
mg	Milligram
min	Minute
ml	Millilitre
Mm	Milli molar
MSV	*Mottle streak virus*
MW	Molecular Weight
MWMV	*Moroccan watermelon mosaic virus*
MYMV	*Mungbean yellow mosaic virus*
N	Normal
NADP-ME	NADP-malic enzyme
NCBI	National Centre for Biotechnology Information

NCM	Nitro cellulose membrane
nm	Nano meter
OD	Optical density
PAb	Polyclonal antibody
PAL	Phenyl alanine ammonialyase
PapMV	*Papaya mosaic virus*
PBS-TPO	Phosphate Buffer Saline -Tween Polyvinyl pyrrolidone ovalbumin
PEPC	Phosphoenolpyruvate carboxylase
PGM	Phospoglucomutase
PGMV	*Peanut green mosaic virus*
PLRV	*Papaya leaf reduction virus*
PO	Peroxidase
PPO	Polyphenol oxidase
PPV	*Plum pox virus*
PRSV	*Papaya ring spot virus*
PStV	*Peanut stripe virus*
PVP	Poly vinyl pyrrolidone
PVY	*Potato virus Y*
Rm	Relative mobility
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcription-Polymerase chain reaction
SDM	Spray dried milk
SDS-PAGE	Sodium dodecyl sulphate-Poly acrylamide gel electrophoresis
SMV	*Sugarcane mosaic virus*
SYMV	*Soybean yellow mosaic virus*
TBS	Tris buffer saline

TEMED	Tetramethyl ethylene diamine
WMV-1	*Water melon mosaic virus*-1
WSMV	*Wheat streak mosaic virus*
wt.	Weight
ZTMV	*Zucchini tigre mosaic virus*
ZYMV	*Zucchini yellow mosaic virus*

Introduction

1. INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp.), belonging to Family Leguminosae is one of the most important food and forage legume in the semi-arid tropics that include parts of Asia, Africa, Southern Europe, Southern United States and Central and South America (Singh, 2005). Cowpea represents a rich source of protein, with 43 and 33 per cent leaf and seed protein content respectively (Nielsen *et al*., 1993). The seeds have a total carbohydrate content ranging from 56 to 68 per cent, the major constituent being starch (45 to 48 per cent) (Longe, 1980).

Annual cowpea production has increased from about 2.40 million tons in 1991, to more than 6.3 million tons in 2008. In India, cowpea is grown in about 0.50 million ha with an average productivity of 600 to 750 kg grains per ha (Singh *et al*., 2012).

Papaya (*Carica papaya* L), Family Caricaceae is an important fruit crop both in tropics and subtropics. The papaya is a good source of calcium, vitamins and proteolytic enzymes. Its protein content is approximately 5 per cent. The nutritional value of the fruit depends on the variety, growing conditions and ripeness upon consumption (Maharaj and Sankat, 1990).

The global production of papaya was 10.5 million tonnes during 2009 to 2010, which has increased to 12.5 million tonnes during 2012 to 2013. India is the largest producer of papaya fruit, with 37 per cent share in world production. In India, the major papaya producing state is Andhra Pradesh, which contributes around 38 per cent of the country's produce (FAO STAT, 2012).

Family *Potyviridae* with its 6 genera, includes the most economically devastating plant viruses. *Blackeye cowpea mosaic virus* (BlCMV), *Cowpea aphid-borne mosaic virus* (CABMV) and *Papaya ring spot virus* (PRSV) comes within the *Potyvirus* genera. The *Potyvirus* genus have non enveloped rod shaped flexuous particles 680-900 nm long and 11-13 nm in diameter, helix pitch 3.4-3.5 nm

encapsidating a genome of about 9.7 kb with multiple copies of a single protein species of 30 to 47 kDa (Riechmann *et al.,* 1992). The genus is mechanically and aphid transmissible.

Cowpea is susceptible to wide range of pests and diseases of which CABMV is a cosmopolitan, economically significant seed borne virus of cowpea. It can cause yield loss of 13 to 87 per cent under field conditions depending upon crop susceptibility, virus strain and the environmental conditions. Infection resulted in leaf mosaic, vein banding, distortion and stunted growth (Bashir *et al*., 2002). Incidence of another aphid transmitted *Potyvirus,* BlCMV have been reported from other parts of India.

PRSV infection results in huge economic losses, with P biotype infecting papaya and W biotype infecting both papaya and cucurbits. PRSV has been recognized as a destructive disease in many tropical and subtropical areas including the USA, South America, Africa, India, Thailand, Taiwan, China, Philippines, Mexico, Australia, Japan, French Polynesia and Cook islands resulting in the decline in fruit production. This disease can cause up to 100 per cent losses of crops in some regions (Tennant *et al*., 2007). In Kerala, disease incidence has increased to 55 per cent (Verma *et al*., 2007). PRSV infection resulted in leaf mosaic, ring spots, shoestring, distorted fruits and stunted growth.

Many varieties have been evolved, showing varied levels of resistance to the *Potyviruses* considered in the present study. Studies on the biochemical basis of resistance and susceptibility can contribute for the development and maintenance of resistance in such varieties. Considering this aspect, the host pathogen interaction studies were undertaken with varieties resistant and susceptible to the *Potyviruses.*

The resistant variety, CO6 from TNAU and susceptible variety, Vellayani Jyothika from Department of Olericulture, College of Agriculture, Vellayani were taken for the study of viral disease in cowpea. Vellayani Jyothika is a high yielding variety of vegetable cowpea (yard-long bean) which is widely cultivated and most

popular among the farmers (KAU POP, 2011). In recent years, high incidence of mosaic disease have been noticed in Vellayani Jyothika, which can be attributed to the high susceptibility of the variety to aphids and seed borne nature of the virus.

The resistant variety, Pusa Nanha from Pusa, New Delhi and a susceptible local variety from Instructional Farm, College of Agriculture, Vellayani were taken for the study of ring spot disease in papaya. The wide spread nature of the disease in the field and other contributing factors have led to the new strains of virus infecting papaya (Chavan *et al*., 2010).

Detailed study on the symptomatology and transmission of *Potyviruses* infecting cowpea and papaya, along with their biological, immunological, molecular detection and characterisation can effectively contribute for the early detection and development of disease management strategies.

Review of Literature

REVIEW OF LITERATURE

Potyviruses

The *Potyviridae* family is one of the largest and economically most important family of plant viruses, due to its effects on crops worldwide. Members of the family are characterized by properties such as vector transmission and particle morphology. The name *Potyviridae* comes from the 'Potato virus Y group'. The *Potyviridae* genome consists of either one molecule or two segments of linear positive-sense single stranded RNA. The complete genome is 8500-10000 nucleotides long. Based on amino acid sequences there are 6 different genera. *Potyvirus* genus is the largest genus with more than 100 species and are transmitted by aphids (Syller, 2006).

Cowpea

Cowpea (*Vigna unguiculata* (L.) Walp.) is an important dual purpose legume in the tropics and subtropics. It is native to Central Africa and is a source of dietary protein, nitrogen fixer, also a fodder crop (Adejumo *et al.,* 2001). The seed is reported to contain 24, 53 and 2 per cent crude protein, carbohydrates and fat respectively (FAOSTAT, 2012). Vegetable cowpea can be grown throughout the year under Kerala conditions. It is a direct sown crop and seed rate varies from four to five kg per ha. It can be grown as a floor crop, an intercrop or a pure crop. Cowpea can be grown in kole lands of Thrissur district during summer where rice crop cannot be raised due to water scarcity.

2.1. Blackeye cowpea mosaic virus (BlCMV) /*Cowpea aphid-borne mosaic virus* (CABMV)

Viral diseases are reported to cause significant reduction in yield of cowpea in Asia, Africa and America. Worldwide, more than 20 viruses have been identified as naturally infecting cowpea (Mali and Thottappilly, 1986) and many are transmitted through seeds (Hampton, 1983). Among the many species of plant viruses infecting cowpea, CABMV is considered to be significant and cosmopolitan, because infection resulted in yield losses between 30 to 40 per cent (Bashir *et al.*, 2002). Lovisolo and

Conti (1966) first reported CABMV from Italy. CABMV has been reported in many countries in different continents including Asia, Africa, Europe, North and South America and Australia (Behncken and Maleevsky, 1977; Mali and Kulthe, 1980; Huguenot *et al.*, 1993; Bashir and Hampton, 1996; Pio-Ribeiro *et al*., 2000). Biological properties of CABMV differed among the isolates worldwide (Bashir *et al.,* 2002).

BlCMV was first reported from the USA (Anderson, 1955; Lima *et al.*, 1979). B1CMV had also been reported in yard-long bean or asparagus bean *Vigna unguiculata* subsp. *sesquipedalis* in the Netherlands (Dijkstra *et al*., 1987).

Mali *et al.* (1988) reported that when 60 cultivars from India were screened for the presence of cowpea viruses, BlCMV was identified from 19 and CABMV from 7, based on transmission and serological tests, particle morphology, host range, reactions of cowpea cultivars and properties in crude sap. Indian isolates of BlCMV showed serological relationships with *Bean common mosaic potyvirus* (BCMV), *Bean yellow mosaic potyvirus* (BYMV) and *Soybean mosaic potyvirus* (SMV), but CABMV showed a relationship with only BCMV. Capsid protein properties of CABMV and BlCMV confirmed their existence as two major subgroups of aphid transmitted legume infecting *Potyviruses* (Mali *et al.,* 1988). CABMV and BlCMV were transmissible through sap, seeds, and by several species of aphids in a non-persistent manner (Brunt *et al.,* 1990). They showed a wide experimental host range and induced characteristic pinwheel inclusions in infected cells.

Based on differential reactions of cowpea genotypes, 21 distinct variants of B1CMV and 25 of CABMV with narrow to wide ranges of pathogenicity and virulence were identified. B1CMV isolates PI-25B5, PU-7B and PU-10B and CABMV isolates CABMV-Mor, RN-18C and PI-39C expressed the highest virulence (Bashir, 1992). Strains of BlCMV were split in to two distinct species: *Bean common mosaic virus* (BCMV) and *Bean common mosaic necrosis virus* (BCMNV) (Vetten *et al*., 1992). Conversely, viruses that were considered as distinct species such as *Peanut stripe virus* (PStV), *Adzuki bean mosaic virus* (AzMV) and BlCMV were

reclassified as BCMV (Mink and Silbernagel, 1992). Puttaraju *et al*. (2000) reported the widespread occurrence of BlCMV in Karnataka, India. BlCMV was found in 18 fields, out of 21 fields surveyed. Disease incidence in the field varied from 1 to 70 per cent. The yield from the infected fields was estimated to be 50 kg when compared to the expected yield of 2500 kg/ha. The infected plants showed severe green and yellow mosaic, vein banding, blistering and leaf roll.

In India, BCMV associated with vanilla (*Vanilla planifolia* Andrews) was characterised based on host reaction and coat protein sequence properties (Bhadramurthy and Bhat, 2009).

2.1.1. SYMPTOMATOLOGY

Bock (1973) described three isolates of CABMV. The first isolate from Kenya, referred to as the 'African strain' induced irregular, angular broken mosaic. Another isolate from East Africa described as 'African vein banding strain', induced broad dark green vein banding. 'African mild strain', the third African isolate, induced very mild mottle with little or no effect on plant growth. Disease symptoms varied with the virus strain and host cultivars but were usually in the form of dark green vein-banding, leaf distortion, blistering and stunting (Bock and Conti, 1974). Sekar and Sulochana (1988) reported that BlCMV infected seedlings showed infection on primary leaves, leaves were crinkled and had dark and light green patches while a few other leaves had yellow patches.

CABMV caused severe diffused chlorotic patches, deformation on leaves and growth stunting (Ward *et al*., 1992). Infection resulted in symptoms comprising dark vein banding or interveinal chlorosis, leaf distortion, blistering and stunting. Under field conditions, CABMV caused yield loss of 13 to 87 per cent (Bashir *et al*., 2002).

Elbeshehy *et al*. (2010) reported that CABMV infection reduced significantly the no. of flowers, length of shoot system, length of root system, volume of root system, no. of bacterial nodules, no. of pods, no. of seeds, total weight of dry pods and total weight of seeds per plant. Amayo *et al.* (2012) reported a significant decrease in plant growth and yield parameters, namely plant height (33.47 per cent),

number of pods per plant (78.87 per cent), number of seeds per plant (81.96 per cent) and grain yield (83.63 per cent) on infection with CABMV.

B1CMV produced both localized and systemic symptoms on cowpea. Localized symptoms were large reddish, often ring-like lesions which typically spread along the veins, forming a reddish-net pattern. Systemic symptoms included mottling and green vein banding along with interveinal chlorosis, stunting and leaf distortion (Thottappilly and Rossel, 1985).

Ribeiro *et al.* (1978) reported that synergistic interaction of CABMV and BlCMV, resulted in stunting of cowpea. Leaves of stunted plants were small, mottled, blistered and malformed. Discoloration varied from different shades of green to greenish yellow particularly with yellowing associated with major veins. The number of leaves were reduced to about 30 per cent and internodes were shortened by 50 per cent or more. Pod set was poor and the number of seed per pod was reduced by 50 to 75 per cent. Huguenot *et al.* (1993) reported that though CABMV and B1CMV produced similar symptoms in their plant host range, they were two different *Potyviruses.*

2.1.2. TRANSMISSION STUDIES

2.1.2.1. Mechanical Transmission

BlCMV was mechanically transmitted to cowpea, which resulted in reddish, necrotic ring spots in inoculated leaves that spread in to network of veinal necrosis. Systemic mosaic was also observed in *Crotalaria spectabilis, Vicia faba* and *Phaseolus vulgaris* (Murphy *et al.*, 1987).

CABMV was mechanically transmitted to cowpea and disease incidence ranged from 8 to 100 per cent for five cowpea cultivars (Ndiaye *et al*., 1993). Elkewey *et al*. (2007) reported that CABMV was mechanically transmitted to 9 of 10 cowpea seedlings (90 per cent). CABMV was mechanically transmissible and systemic infections were observed in *V. unguiculata* subsp. *unguiculata, V. unguiculata* subsp. *sesquipedalis*, and *Nicotiana benthamiana* (Damiri *et al.*, 2012).

2.1.2.2. Seed Transmission

Viral diseases of cowpea have been reported to cause appreciable losses in yield if the plants are infected in early growth stages (Booker *et al.,* 2005).

A sample of 158 *Vigna unguiculata* plant introductions and germplasm accessions was tested for seed borne *Potyviruses.* Of these, 21 accessions were found to be infected either with B1CMV (0.0 to 6.9 per cent) and CABMV (0.0 to 13.3 per cent) *Potyviruses*. The highest seed transmission rates of 48.5 and 55 per cent were produced with B1CMV isolate PI-25B1 and CABMV isolate RN-27C respectively (Bashir, 1992).

Bashir and Hampton (1996) reported natural seed transmission incidence of 0 to 6.9, 0 to 13.3 and 0 to 2.0 per cent for BlCMV, CABMV and *Cucumber mosaic cucumovirus* (CMV) respectively. Udayashankar *et al.* (2010) opined that seed borne transmission of CABMV recorded a frequency rate of 30 per cent. The seed transmission levels of 23.0, 20.3 and 16.4 per cent were recorded for CMV, *Cowpea mild mottle calarvirus* (CPMMV) and CABMV respectively. (Amayo *et al*., 2012)

2.1.2.3. Insect Transmission

CABMV was efficiently transmitted by *Aphis craccivora* in a non-persistent manner (Bock and Conti, 1974; Harrison and Robinson, 1988; Damiri *et al.,* 2012), although other aphid species, *i.e*. *Myzus persicae* (Sulzer), *Aphis citricola* van der Goot. (Lovisolo and Conti, 1966; Fischer and Lockhart, 1976), *Aphis gossypii* Glov. and *Toxoptera citricidus* Kirk. (Gillaspie *et al.,* 2001) were also reported as vectors.

Three species in the genus *Aphis* (*A. craccivora*, *A. spiraecola* and *A. gossypii*) transmitted CABMV efficiently to and from cowpea, bean and soybean host plants. *Rhopalosiphum maidis* and *Cerataphis palmae* were also reported to transmit the virus, but less efficiently. *R. maidis* and *C. palmae* caused primary loci of infection and also brought about limited secondary spread. However, colonizing *Aphis* spp. were mainly responsible for secondary spread within the field. (Atiri *et al*., 1986). Murphy *et al.* (1987) reported non persistent transmission of BlCMV by *Myzus euphorbiae*.

Bashir and Hampton (1994) reported a non-persistent aphid transmission for BlCMV and CABMV using 3 aphids (*A. craccivora*) per cowpea plant. Transmission rates ranged from 24 to 55 and 18 to 57 per cent for the viruses respectively. Elkewey *et al*. (2007) reported that *M. persicae* could transmit CABMV to 60 per cent of inoculated cowpea plants.

2.1.3. HOST PATHOGEN INTERACTION STUDIES

Virus infection generally resulted in the drastic biochemical and physiological changes in the host plants, which could be used to characterise the viruses causing the disease along with symptomatology (Herbers *et al.,* 2000).

2.1.3.1. Estimation of Total Carbohydrate

Thind *et al.* (1996) reported that the amount of reducing sugars, non reducing sugars, total sugars and starch decreased in black gram infected with *Blackgram yellow mosaic virus* (BGYMV). Mali *et al*. (2000) reported that BGYMV infection on moth bean (*Vigna aconitifolia*) resulted in reduction of total soluble carbohydrates in susceptible variety.

Production of sucrose and total carbohydrates was significantly reduced in *Soybean yellow mosaic virus* (SYMV) infected plants (Xiaoyan *et al*., 2000). Hemida (2005) reported that infection of *Bean yellow mosaic virus* (BYMV) on *Phaseolus vulgaris*, reduced the carbohydrate level by 79 per cent. Sinha and Srivastava (2010) reported reduced carbohydrate content (total sugars and starch) in mungbean plants infected with *Mungbean yellow mosaic virus* (MYMV).

2.1.3.2. Estimation of Chlorophyll

The reduction in total chlorophyll due to BYMV on beans, SYMV on soyabean and *Peanut green mosaic virus* (PGMV) on groundnut was 39, 60 and 36 per cent respectively (Chinnadurai and Nair, 1971; Narayanasamy and Palaniswami, 1973; Naidu *et al*., 1984).

Irvine (1971) studied on the effect of four strains of Sugarcane mosaic virus (SCMV) *viz.*, A, B, D and I on plant photosynthesis efficiency. All the four strains decreased the rate of photosynthesis per unit area, total chlorophyll content and

increased the amount of light transmitted by the leaf. Mayoral *et al.* (1989) reported that the *Cowpea mosaic virus* (CPMV) infection reduced the chlorophyll content of infected plants, but content of carotene remained unchanged.

Radhika and Umamaheswaran (2000) reported higher chlorophyll content in resistant variety of cowpea (CO6) when compared to susceptible variety (Sharika), on infection with BlCMV. At 20 days of infection, the leaves of BYMV infected *Phaseolus vulgaris*, showed a reduction of 50, 50 and 40 per cent in chlorophyll a, chlorophyll b and carotenoids contents respectively (Hemida, 2005).

Arora *et al*. (2009) reported that, BGYMV infection in mothbean caused significant reduction in chlorophyll content of the infected leaf tissues. The influence of viral infection caused by two different *Potyviruses*, *Potato virus Y* (PVY) *and Potato virus A* (PVA) on plant metabolism and photosynthetic apparatus of *Nicotiana tabacum* L. cultivars Samsun and Petit Havana SR1 were studied. Viral infection, namely PVY, affected more negatively the photosynthetic apparatus of cultivars Petit Havana SR1 and Samsun (Ryslava *et al.*, 2013).

2.1.3.3. Estimation of Phenol

Ramiah (1978) reported that, there was no difference in phenol content between healthy and CABMV infected leaves of resistant cultivars, MS 9804 and C0-1. Dantre (1983); Singh and Srivastav (1999) reported that CABMV virus infection increased the total phenols in diseased leaf compared to healthy ones.

Radhika (1999) opined that there was not much difference in phenol content between resistant and susceptible genotypes on inoculation with BlCMV. Mali *et al.* (2000) recorded a higher phenol content in BGYMV infected moth bean leaves. Sinha and Srivastava (2010) reported a loss of total chlorophyll, chlorophyll a and chlorophyll b content due to MYMV infection.

Shilpasree *et al.* (2012) investigated on changes in phenol content between resistant and susceptible genotypes of cowpea, DCS-6 and GC-3 respectively. With increase in age of the plant from 30 to 60 days, the phenol content increased (0.51 to 0.57 mg per g fresh weight) in the susceptible genotype, whereas, there was depletion of total phenols (0.71 to 0.52 mg per g fresh weight) in the resistant genotype. No statistically significant differences were observed in total phenol and flavonoid levels between healthy and CABMV infected passion fruit plants (Tomomitsu *et al*., 2014).

2.1.3.4. Estimation of Total Soluble Protein

Singh (1982) while investigating changes in nitrogenous constituents of cowpea pods, found that due to CPMV infection, there was increased total nitrogen, protein and nitrate nitrogen. Thind *et al.* (1996) reported that MYMV infection on mung bean lowered the protein content in leaves. Mali *et al.* (2000) reported that free aminoacid and soluble protein content increased with increased levels of MYMV infection on susceptible varieties of mungbean.

Sindhu (2001) reported that BlCMV infection resulted in increased total soluble protein content in resistant genotype. The leaves of BYMV infected *Phaseolus vulgaris* showed a reduction in amino acid content of 50 and 77 per cent at 12 and 20 days after infection, respectively (Hemida, 2005). Langhams and Glover (2005) reported, 0.2 to 18.5 per cent increase in seed protein of winter wheat inoculated with *Wheat streak mosaic virus* (WSMV).

Taiwo *et al.* (2007) demonstrated that individual infection with CABMV, *Cowpea mottle virus* (CPMoV) and *Southern bean mosaic virus* (SBMV) as well as mixed infection, lowered the protein content of the seeds of all the cowpea cultivars and lines by 24.8 to 28.9 per cent. Sinha and Srivastava (2010) indicated an increase in total protein content (1.64 to 1.99 mg per 100 mg dry weight) in MYMV infected mungbean plants than their healthy counterparts (1.11 to 1.59 mg per 100 mg dry weight). Mohammed (2011) reported that *Beet mosaic virus* (BtMV) inoculated Beet plants (*Beta vulgaris* L.) recorded 7.93, 8.53, and 19.27 per cent increase in protein

content over healthy plants at first, second and third week respectively after inoculation.

2.1.3.5. Estimation of Defense Related Enzymes

Sohal and Bajaj (1993) reported an increase in polyphenol oxidase activity in resistant variety of mung bean infected with MYMV. Umamaheswaran (1996) reported that there was progressive increase in peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonialyase (PAL) activity in inoculated and susceptible varieties of cowpea. Yunzhu *et al.* (1997) and Cuiming *et al.* (1997) observed that resistant cultivars on inoculation with SMV showed an increased activity of PO and PPO. Ming *et al.* (1999) observed that, after inoculation with SMV, peroxidase activity increased a little in the susceptible varieties, but both peroxidase and polyphenol oxidase activities were rapidly decreased in the seed coat of resistant varieties. Superoxidase activity increased in susceptible varieties after inoculation with SMV, while it remained stable in the resistant varieties. Mali *et al.* (2000) reported that the activity of catalase, PO and nitrate reductase decreased, with increased intensity of disease in case of Yellow mosaic disease of moth bean. Radhika and Umamaheswaran (2000) reported a higher activity of PO, PPO and PAL in resistant variety when compared to susceptible variety of cowpea.

2.1.3.6. Electrophoretic Analysis of Proteins using SDS-PAGE

Lima *et al.* (1979) reported that by the method of Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE), inclusions of BlCMV consisted of a single protein with molecular weight (MW) of 70 kDa whereas, freshly purified BlCMV had a protein with MW of 34 kDa and 2 small proteins with MW of 29 kDa and 27 kDa. Taiwo and Gonsalves (1981) conducted SDS-PAGE on SDS dissociated virus preparations and reported that, capsid protein of CABMV had MW of 34,400. Analysis of purified isolate of BlCMV (BlCMV-AC) from alyce-clover (*Alysicarpus vaginalis*) by SDS-PAGE, revealed a major and a minor protein component with relative MW of 34.5 and 31 kDa respectively (Zhao *et al*., 1991).

SDS-PAGE of extracts from leaves infected with BCMV strains showed that the apparent MW's of the capsid protein of the serotype A and B isolates were 33 and 35 kDa respectively (Vetten *et al*., 1992). Ayisha (2005) performed protein profile studies on healthy and *Tospovirus* inoculated cowpea plants. Experiment revealed three newly induced virus related proteins in inoculated plants with MW's of 28, 15 and 6.2 kDa.

Sousa *et al.* (1996); Elkewey *et al*. (2007) reported that a large MW of 35 kDa was observed when CABMV purified virus preparation was subjected to SDS-PAGE. Bhadramurthy and Bhat (2009) reported that partially purified BCMV subjected to SDS-PAGE, revealed a major band corresponding to 34 kDa.

2.1.3.7. Electrophoretic Analysis of Isozyme

Isozyme analysis is a powerful tool for estimating genetic variability in identifying cultivars and germplasm accessions (Asiedu, 1992).

Farka and Stahman (1966) conducted native-PAGE and reported the appearance of 2 new PO's (peroxidases II and III) at the site of lesion formation due to SBMV. Umamaheswaran (1996) indicated that there was significant variation in PO isozyme in resistant and susceptible cultivars of cowpea. Sindhu (2001) conducted electrophoretic analysis of isozymes from CABMV susceptible cowpea varieties and found 5 isoforms of PPO in susceptible varieties, Sharika and Malika, whereas CO6 and Pallichal local expressed only 4 isoforms of PPO.

The difference in the isozyme pattern can be used in the biochemical characterization of resistance and susceptibility of host plant to CABMV. Shilpasree *et al.* (2012) reported that, in resistant genotype, DCS-6, Rm values of healthy leaf were 0.40, 0.42 and 0.43 and that of the diseased leaf were 0.46, 0.48 and 0.50. In susceptible genotype GC3, Rm values of healthy leaf were 0.53, 0.51 and 0.52 and that of the diseased leaf were 0.65, 0.58 and 0.59.

2.1.4. BIOLOGICAL DETECTION

By utilising the method of bio-assay in *Chenopodium amaranticolor*, characterisation of three strains of CABMV (type, vein banding and mild) was done

(Bock, 1973). Khatab and Eman (2002); Elkewey *et al.* (2007) reported that CABMV induced chlorotic, local lesions on inoculated leaves of *Chenopodium quinoa*.

CABMV induced local and systemic symptoms in *C. quinoa, Dolichos biflorus, Nicotiana occidentalis, Phaseolus aborigineus, P. lathyroides* and several cultivars of *P. vulgaris*. It induced systemic symptoms in *Cajanus cajan*, *Canavalia ensiformis*, *Cassia occidentalis*, some *Crotalaria* sp., *N. benthamiana* and *P.lunatus* (Ladipo *et al.,* 2004). Damiri *et al*. (2012) reported on the development of local infections on CABMV inoculated leaves of *C. quinoa, C. amaranticolor,* and *N. occidentalis*. Also latent infections were detected in inoculated *Ocimum basilicum* and *Cajanus cajan.*

2.1.5. IMMUNOLOGICAL DETECTION

2.1.5.1. Enzyme linked immunosorbent assay (ELISA)

On the basis of ELISA results, virus isolates of BICMV and CABMV were identified from germplasm of yard-long bean (*Vigna unguiculata* ssp.*sesquipedalis*) and soybean plants (*Glycine max*) (Dijkstra *et al*., 1987). Eighteen isolates of BCMV, five isolates of B1CMV, four isolates of CABMV, and one isolate each of AzMV, and PStV were compared serologically using a panel of 12 monoclonal antibodies (MAbs), in indirect ELISA. Four MAbs detected all virus isolates, one detected all isolates except those of CABMV. Three MAbs were specific only for serotype A isolates of BCMV. Four MAbs detected all serotype B isolates of BCMV along with the isolates of B1CMV, AzMV and PStMV. None of the antibodies distinguished among these four viruses (Mink and Silbernagel, 1992).

Ndiaye *et al*. (1993); Bashir and Hampton (1996) used Direct antigen coating-ELISA (DAC-ELISA) and Double antibody sandwich-ELISA (DAS-ELISA) for detection of seed borne viruses in cowpea seeds. Konate and Neya (1996) reported that a biotin/ streptavidin ELISA technique was found to be more sensitive than a standard ELISA protocol for detecting CABMV infection in cowpea seeds. The method could detect 1 CABMV infected seed in a group size of 500 seeds. The

serological methods using polyclonal (PAb) and monoclonal antibodies are efficient analytic tools for the detection of BICMV for screening and virus control purposes (Hao *et al.,* 2003).

Ladipo *et al.* (2004) identified a strain of CABMV designated as CABMV-Cr using the serological technique, ELISA with BlCMV and CABMV antibodies. The coating MAbs and the detecting biotinglated MAbs were used in the following combinations: 5H5-6F9, 5H5-3G9, 4B12-4B12, 12F9-6C10, 5H5-7D9, 1F5-1F5, 15E6-10G5 and 16G5-10G5. Taiwo *et al*. (2007) reported that the age of plant at the time of infection and type of cultivar had significant effect on the titre of CABMV in the infected plants, with highest titre (0.11 to 0.46) detected by ELISA, 10 days after planting.

Samples collected from symptomatic cowpea plants grown in the field gave positive reactions with CABMV antibodies but not with BlCMV antibodies in DAS-ELISA, thus confirming that the virus infecting cowpea in Kingdom of Saudi Arabia was CABMV (Damiri *et al.,* 2012). Amayo *et al.* (2013) used DAS-ELISA to analyse the seed and leaf samples from CABMV symptomatic and asymptomatic plants. Serological test by Plate trapped antigen-ELISA (PTA-ELISA) with group specific antiserum, at a dilution of 1:1500 confirmed the presence of *Potyvirus* infecting cowpea (Sharma *et al*., 2013)

2.1.5.2. Dot immunobinding assay (DIBA)

DIBA has been used for CABMV identification in cowpea plants (Sidaros *et al.,* 2006; Akinjogunla *et al.,* 2008; Damiri *et al.,* 2012) and it could detect the virus in sample extracts diluted up to 1:1000 (El-Kewey *et al.,* 2007).

Leaf samples collected from naturally infected cowpea and from those infected via seeds gave positive dot blot hybridization responses while healthy controls were negative (Damiri *et al.,* 2012).

2.1.6. MOLECULAR DETECTION AND CHARACTERISATION

2.1.6.1. Reverse transcription-Polymerase chain reaction (RT-PCR)

A universal primer (5′-GGX AAY AAY AGY GGX CAZ CC-3′, X = A, G, C or T; Y = T or C; Z = A or G), designed from the consensus sequences that code for the conserved sequence GNNSGQP in the NIb region of members of the family *Potyviridae*, was used to amplify by RT-PCR, the 3′-terminal genome regions from infected plant samples representing 21 different viruses in the family *Potyviridae* (Chen *et al*., 2001). Gillaspie *et al*. (2001) reported that RT-PCR detected more virus in seeds infected with CABMV than in the number of infected seedlings normally arising in germination tests. RT-PCR was also extremely sensitive and detected 1 infected leaf among 99 healthy leaves, while ELISA detected only one infected leaf among nine healthy leaves.

Elkewey *et al*. (2007) used RT-PCR to get a 345 bp size product of coat protein gene of CABMV. Wei *et al*. (2009) reported that a 25 mer oligonucleotide microarray was feasible for the simultaneous detection of multiple *Potyviruses.* Using RT-PCR with virus genus and family specific degenerate primers, CABMV, *Cucumber mosaic virus* (CMV) and SBMV were identified (Salem *et al.,* 2010). RT-PCR technique was used for studying the homology between CABMV and its closely related virus *Cowpea severe mosaic virus* (CPSMV) (Abreu and Medeiros, 2012).

BlCMV coat protein gene (BICMV-CP) was amplified at 864 bp from naturally infected cowpea and yard-long bean using CACP1 forward primer and CACP2 reverse primer (Koohapitagtam and Nualsri, 2013). Sharma *et al*. (2013) amplified a 723 bp DNA fragment from CABMV infected plants by RT-PCR using a pair of degenerate primers specific for coat protein and 3’ UTR.

2.1.6.2. Characterisation

Niang *et al*. (1996) reported that Zimbabwe isolate of CABMV comprised of an open reading frame (ORF) of 990 nucleotides and a 3' non-coding-region of 231 nucleotides followed by a poly-A. The ORF had high similarity to NIb and CP of

Potyviruses. Boxtel *et al*. (2000) reported that the level of nucleotide identity of CABMV strains varied from 83 to 97 per cent, but mostly between 84 to 89 per cent.

Full genome sequencing of BlCMV-Taiwan strain (BICMV-Tw) and its phylogenetic analysis with BCMV-Y, BCMV-R, PStV, SMV, CABMV, *Clover yellow vein virus* (ClYVV), *Bean yellow mosaic virus* (BYMV) and *Lettuce mosaic virus* (LMV) was carried out (Wang and Fang, 2004). The genome consisted of 9992 nucleotides composed of 5' UTR, an ORF encoding a polyprotein of 3202 amino acids, 3' UTR and poly (A) tail, respectively. Maximum homology of BICMV-Tw sequence (80 per cent) was observed with BCMV-Y, BCMV-R and PStV except P1 region. Nascimento *et al.* (2006) compared sequences of virus infecting passion fruit with those of other *Potyviruses* and indicated the highest identity with CABMV isolates (85 to 94 per cent). Thus confirmed that passion fruit woodiness disease was primarily caused by CABMV.

Bhadhramurthy and Bhat (2009) reported that BCMV isolate infecting vanilla showed 87 to 96 and 87 to 98 per cent identities at nucleotide and aminoacid level with other BCMV isolates. The isolate showed maximum identity of 96 and 98 per cent at nucleotide and amino acid level, respectively, with Blackeye (Blk1) strain of BCMV indicating its closeness to cowpea infecting BCMV isolates. Damiri *et al.* (2012) identified a new strain of CABMV (CABMV-SA) from Saudi Arabia.

Sharma *et al*. (2013) reported that CABMV isolate CmRp (KC753448) was closely related to a Zimbabwe isolate, CABMV-Z[ZB] (AF348210) The CABMV isolate also showed 99 and 81 per cent identity with Indian isolate, BCMV-NL-4[IN] (JN692258). The coat protein of 10 CABMV isolates (CABMV-Lns1-CABMV-Lns10) from, Bahia State, Brazil, were sequenced and presented very close identity between themselves (nucleotide: 97 to 99 per cent, amino acid: 95 to 100 per cent). Though, they were phylogenetically closely related to Brazilian CABMV, were found to form an isolated cluster within the Brazilian clade (Melo *et al.,* 2015).

PAPAYA

Papaya (*Carica papaya* L), Family Caricaceae, is an important fruit crop both in tropics and subtropics, because of its high nutritive and medicinal value. *C. papaya* originated from the lowlands of eastern Central America, Mexico to Panama (Nakasone and Paull, 1998). The global papaya production has grown significantly over the last few years, the biggest increase was between 2009 and 2010, as production in India increased by 20.50 per cent. Other leading producers are Brazil, Mexico, Nigeria, Indonesia, China, Peru, Thailand and Philippines (FAOSTAT, 2012). Papaya is a powerhouse of nutrients and is available throughout the year. It is a rich source of three powerful antioxidants (vitamin C, vitamin A and vitamin E), minerals, B vitamin pantothenic acid and fibre (Aravind *et al.,* 2013).

Papaya thrives well in tropical climate. The occurrence of low temperature and frost limits its cultivation. In Kerala, the limiting factors for commercial cultivation are high rainfall and severe drought in summer. However, this is best suited as a homestead fruit crop (KAU POP, 2011).

2.2. Papaya ring spot virus (PRSV)

Viruses belonging to 6 taxonomic groups can infect and induce diseases of varying economical importance in papaya but *Papaya ring spot virus* (PRSV) is by far the most serious of the virus diseases (Fermin and Gonsalves, 2003). Due to its wide distribution, PRSV is a major limiting factor for papaya production throughout the tropics and subtropics (Gonsalves, 1998).

Jensen (1949); Webb and Scott (1965) first described PRSV in papaya (PRSV-P) and cucurbits (PRSV-W) respectively. The virus has since been recognized in many tropical and subtropical areas including the USA, South America, Africa (Costa *et al*., 1969; Purcifull *et al*., 1984), India (Khurana, 1974), Mexico

(Alvizo and Rojkind, 1987), Thailand, Taiwan, China, Philippines (Gonsalves, 1994), Australia (Thomas and Dodman, 1993) and Japan (Maoka *et al.,* 1995).

PRSV-W was considered for a long time to be a distinct *Potyvirus*, WMV-1 *Water melon mosaic virus*-1 (WMV-1) (Webb and Scott, 1965). However, the P and W isolates were found to be indistinguishable serologically (Gonsalves and Ishii, 1980) and now are considered strains of PRSV (Purcifull *et al*., 1984; Suzuki *et al.,* 1990; Baker *et al.,* 1991). The main reason for the early confusion about the taxonomic status of the W isolate was that it does not infect papaya. However, P isolates infected cucurbits as well as papaya (Provvidenti, 1993; Tennant *et al*., 1994).

PRSV particles are flexuous filaments of 780nm×12nm, that contained a single-stranded RNA genome of positive polarity with a single open reading frame (ORF) encoding a polyprotein *i.e.* subsequently processed by viral-encoded proteinases P1, HC-Pro and NIa (Yeh *et al.,* 1992). The genomic RNA consisted of 10,326 nucleotides and has the typical array of genes found in *Potyviruses* (Shukla *et al.,* 1989).

PRSV-P was thought to have arisen by mutation from PRSV-W. PRSV may have arisen in Asia, in the region of the Indian subcontinent (Sri Lanka) (Bateson *et al.,* 2002). PRSV-P has been isolated from most papaya-producing areas of the world including Africa, tropical Asia, Caribbean, Central and South America and the South Pacific (Tennant *et al.,* 2007). The incidence of PRSV reached 3 to 100, 35 to 66, 75 to 90, 55, 60, 4 to 90 and 40 per cent in Maharashtra, Madhya Pradesh, Bihar, Kerala, Karnataka, Uttar Pradesh, and West Bengal respectively. The most susceptible cultivar was Red Lady. The disease incidence in various cultivars varied from 3 to 100 per cent (Verma, 2007).

PRSV is a highly variable virus species (Bateson *et al*., 2002) and the observed genetic variability is somehow related to the geographical origin of the isolates from large populations rather than smaller isolated populations (Silva-Rosales *et al.,* 2000; Jain *et al.,* 2004). Gibbs *et al.* (2008) provided evidence on the

Asian origin of PRSV and proposed that the introduction of the virus to the Americas occurred some 300 years ago.

PRSV-W incidence was reported from bottle gourd (Mantri *et al*., 2004), gherkin (Rashmi *et al*., 2005), sponge gourd (Verma *et al.,* 2006) and snake gourd (Kumar *et al*., 2014) from India.

Laney *et al*. (2012) identified mixed infection of PRSV and WMV-1 in the black locust (*Robinia pseudoacacia* L.). Romay *et al.* (2014) described a third type of PRSV, *Zucchini tigre mosaic virus* (ZTMV) in addition, to the two types of PRSV.

2.2.1. SYMPTOMATOLOGY

Isolates within type-P differed in the severity of the symptoms produced. Symptom expression is highly influenced by environmental conditions. Symptoms were more severely expressed during cooler months (Gonsalves and Ishi, 1980). The virus caused a range of symptoms including mosaic and chlorosis in the lamina, water-soaked oily streaks on the petiole and upper part of the papaya trunk, distortion of young leaves and ring spot on the papaya fruit (Gonsalves, 1998). Effects of the disease include decreased leaf canopy size and stunting of the plant (Gaskill *et al*., 2002).

Rahman *et al.* (2008) studied on the 7 distinctly defined symptoms caused by 7 PRSV isolates from Bangladesh, namely, mild mosaic, mosaic, fern leaf, severe mosaic, vein clearing, leaf distortion and chlorotic leaf spot. Petioles and tender parts of plant showed oily streaks. The reduction in plant height, canopy diameter, bearing stem and stem girth ranged from 11.86 to 34.07, 13.94 to 46.22, 9.92 to 49.46 and 9.28 to 34.02 per cent respectively and such reduction varied from 17.02 to 48.94, 19.97 to 82.28, 22.36 to 71.59, 35.36 to 82.34 per cent in fruit number, fruit size, fruit weight and yield per plant respectively due to infection with seven different symptomatic isolates of PRSV-P. The development of circular or concentric water soaked lesions and necrotic rings with a solid central spot on mature green fruits were the most characteristic diagnostic symptoms of the disease (Rao *et al*., 2008).

The most commonly observed symptoms of PRSV-W strain were severe mosaic, leaf distortion, oily streaks or spots on papaya; leaf distortion, blisters and shoe stringing on zucchini; and mosaic or yellow mosaic, blisters, and leaf distortion on other cucurbits. Average incidence of plants with symptoms ranged from 75 to 100 per cent on papaya; 85 to 100 per cent on zucchini; 4 to 100 per cent on cucumber; 4 to 100 per cent on pumpkin and 10 to 100 per cent on bottle gourd, choyote and watermelon (Dahal *et al.*, 2008).

Singh and Shukla (2009) reported that the average yield loss suffered by the papaya plant was 61.3 per cent. The economic value of the infected host was reduced to only 38.7 per cent. Gonsalves *et al.* (2010) reported that trees which were infected at a young stage remain stunted and did not produce an economical crop. Fruits from infected trees had bumps similar to those observed on fruit of plants with boron deficiency. A severe PRSV isolate from Taiwan was also known to induce systemic necrosis and wilting along with mosaic and chlorosis. Singh and Shukla (2012) reported that PRSV induced ring spot, distortion and mottling symptoms on foliage, stunting in stem, fragile roots and deteriorated fruit quality and quantity. Plants showed crowding of top leaves and denuded appearance due to defoliation, reduced fruiting and severe stunting.

Papaya plants exhibiting symptoms such as multiple axillary shoots, leaf reduction, mosaic, chlorosis, flattened petiole, oily spots on stem and virescence of flowers were observed in Pune (India) (Verma *et al.*, 2014). It was the first report of a mixed infection from PRSV and phytoplasma.

2.2.2. TRANSMISSION STUDIES

2.2.2.1 Mechanical Transmission

PRSV was mechanically transmitted to papaya seedlings and transmission rate of 60 per cent was recorded (Bayot *et al.*, 1990). Among the many factors which influenced the mechanical transmission, the most important factor was the physiological state of plant at time of inoculation. Salazar (1999) indicated that some

inhibitors may bind to viral particle, but the mechanism of their action was not cleared.

Kelaniyangoda and Madhubashin (2010) reported that 80 per cent of the papaya plants gave positive value in the indirect ELISA, during mechanical transmission study. An isolate of virus (PRSV-P-B) from papaya (Batangus, Philippines) was mechanically inoculated and found to be infectious to 7 species of plants in 3 families including papaya, zucchini (*Cucurbita pepo)*, two types of melons (*Cucumis melo, Cucumis melo var. conomon*), pumpkin (*Cucurbita moschata*) and cucumber (*Cucumis sativus*) (Natsuaki, 2011).

2.2.2.2. Seed Transmission

Bayot *et al*. (1990) reported that two of 1355 seedlings (0.15 per cent) from the fruit of an infected tree developed symptoms of PRSV six weeks after emergence. Reddy *et al.* (2007) conducted seed transmission studies of PRSV and reported that the virus was not seed borne. Laney *et al.* (2012) identified black locust isolates of PRSV and WMV were seed borne (50 per cent transmission respectively).

2.2.2.3. Insect Transmission

The mode of PRSV transmission was characterised by a short acquisition period followed by rapid loss of infectivity of aphid vectors (Purcifull *et al*., 1984). Unstarved apterous adults of *Aphis craccivora* transmitted the PRSV by 25 per cent. Pre-acquisition starvation increased the efficiency of the vector. Transmission increased with an increase in the number of aphids and maximum transmission was recorded with 8 nymphs and 8 apterae adult aphids per test plant (Taya and Singh, 1997).

Single aphid inoculation studies indicated that *Myzus persicae* (56 per cent) and *Aphis gossypii* (53 per cent) were significantly more efficient in transmitting PRSV than *A. craccivora* (38 per cent). PRSV transmission efficiency was 100 per cent in all three species, when a group of five aphids were used per plant (Kalleshwaraswamy and Kumar, 2008). Kumar *et al*. (2010) reported a significant

decrease in PRSV transmission with increased sequestration periods in the three species of aphids.

Singh and Singh (2010) identified *M. persicae* was the most efficient vector (transmitting 70 per cent disease within 12 days after inoculation) of PRSV. It could acquire the virus without any pre-acquisition fasting and showed a decline in transmission after 4 h of pre-acquisition fasting. It could acquire the virus in just 30 sec of acquisition feeding with optimum at 3 min. The aphid readily transmitted PRSV after 2 min of feeding with an optimum transmission after 6 min of feeding. Omar *et al*. (2011) reported that *M. persicae* was able to transmit 8 Egyptian PRSV isolates from infected squash plants to healthy squash plants in a non-persistent manner with 60 sec acquisition and 1 h inoculation periods with a recorded transmission of 100 per cent.

2.2.3. HOST PATHOGEN INTERACTION STUDIES

Multiplication of virus particles in the infected plant cells altered biochemical compounds of cells such as chlorophyll, β-carotene, organic carbon, nucleic acids etc. (Fraser, 1987). External manifestations of disease symptoms were the results of altered host metabolism. The extent of crop loss was mainly associated with the severity of visible symptoms (Sreenivasulu *et al.,* 1989).

2.2.3.1. Estimation of Total Carbohydrate

Mosaic type of virus diseases reduced the carbohydrate contents in infected plants (Dunlap, 1930).

Khatri and Chenulu (1969) reported that carbohydrate content decreased due to *Papaya leaf reduction virus* (PLRV) infection. Singh (1973) reported that PLRV infection resulted in decreased reducing sugar, non-reducing sugar and starch at all intervals after inoculation (5, 10, 15, 20, 25 and 30 days after inoculation) in the variety, Washington. Herbers *et al*. (2000) reported an accumulation of soluble sugars, an induction of cell wall invertase and a gradual decrease in the sucrose to hexose ratio in potato leaves, four days after *Potato Virus Y* (PVY) infection.

Singh (2002) reported that PRSV infection decreased total carbohydrate content in papaya leaves. Kunkalikar *et al*. (2006) identified moderate level of polysaccharides content in PRSV diseased leaves compared to the rich content in healthy leaves. Carbohydrate content was found to be reduced in PRSV infected tissue for reducing sugars (13.60, 8.13 and 5.25 mg per 100 mg dry weight), non-reducing sugars (8.43, 5.88 and 2.63 per cent) and starch content (15.41, 11.31 and 6.57 per cent) in leaf, stem and root samples, respectively (Singh and Shukla, 2009).

2.2.3.1. Estimation of Chlorophyll

Chlorophyll content per unit leaf area was reduced at all three stages of infection by PRSV at early infection, the stage of maximum symptom expression and the recovery phase of systemic disease development, with the greatest reduction coinciding with the phase of maximum symptom expression. Net photosynthesis was also reduced over a range of light intensities (Naidu *et al.,* 1984). Chlorophyll content of banana was found to be reduced at all stages of infection by Banana bract mosaic virus (BBrMV) (Dhanya, 2004). Rahman *et al.* (2008) reported that all the 7 symptomatic isolates of PRSV from Bangladesh, decreased chlorophyll a, chlorophyll b, total chlorophyll and carotenoid pigments in the variety, Shahi.

Radwan *et al.* (2007) recorded that *Zucchini yellow mosaic virus* (ZYMV) infection on pumpkin leaves, reduced the chlorophyll a, chlorophyll b and carotenoid contents by 48, 53 and 52 per cent respectively. Muqit *et al*. (2007) reported that mixed infection of *Bottle gourd mosaic virus* (BgMV), WMV2 and PRSV on ash gourd (*Benincasa hispida*) resulted in increased ratio of chlorophyll a to b and reduced carotene content. Highest reduction was observed in BgMV (78.59 per cent) followed by PRSV (37.39 per cent) and WMV2 (8.86 per cent). The total chlorophyll, chlorophyll a, chlorophyll b and carotenoid content were lower in PRSV infected tissue (67.5, 38.7, 2.7 and 0.3 per cent respectively) (Singh and Shukla, 2009). Mederos *et al.* (2009) reported that PRSV (isolate PRSV-VC) infected papaya samples, recorded significant differences in chlorophyll a, chlorophyll b and total

chlorophyll contents compared to healthy control. They also observed a higher content of chlorophyll a, than chlorophyll b in 5:1 ratio.

2.2.3.2. Estimation of Phenol

Kato *et al*. (1993) extracted and characterized 2 phenolic compounds from cowpea leaves infected with CMV. The reported a higher total phenol content in virus infected leaves of cowpea plants. Smitha, 2001); Dhanya (2004); Aliya (2014) reported an increased phenol accumulation due to BBrMV infection. Saveetha *et al.* (2010) recorded a higher phenol content in finger millet plants infected by *Mottle streak virus* (MSV).

2.2.3.3. Estimation of Total Soluble Protein

Elberthagen (1958); Singh *et al*. (1979); Sun (1983) reported that PRSV infection increased total protein content in papaya leaves.

Mathur and Shukla (1976) reported an increase in the contents of proline, glutamic acid and methionine in the PRSV infected papaya leaves. They found a decrease in the contents of leucine, lysine, threonine and hydroxyproline while cysteine and tyrosine levels remained unchanged. Wijeendra *et al.* (1995) reported that the contents of seven amino acids (alanine, serine, glycine arginine, aspartic acid, threonine and lysine) were increased up to two fold in the PRSV infected leaves. The contents of valine, histidine, methionine and tyrosine were decreased while leucine, isoleucine, phenylalanine and glutamic acid remained unchanged in both naturally infected and mechanically inoculated plants.

Muquit *et al.* (2007) reported that percentage of carbon, nitrogen and protein contents in ash gourd were decreased due to infection with WMV-2 and BgMV, while the contents increased due to PRSV infection. Proline content of ZYMV infected plants were found higher than controls (Radwan *et al.*, 2007). Nitrogen and

protein contents were higher in PRSV infected tissue compared to the healthy counterparts (3.7 and 10.1 per cent respectively) (Singh and Shukla, 2009).

2.2.3.4. Estimation of Defense Related Enzymes

Shaw *et al.* (1994) reported that PRSV resistant and tolerant cultivars had lower PPO activities than virus susceptible cultivars. Chang and Yan (2006) recorded higher activities of PO and PPO in the natural resistant mutant of PRSV compared to susceptible cultivar Suizhonghong, also activities increased at early stage and then declined.

Plum pox virus (PPV) infection brought about, a decrease in apoplastic PO and superoxide dismutase activities, whereas a strong increase in polyphenol oxidase in the susceptible apricot cultivar Real Fino. However, in the resistant apricot cultivar, Stark Early Orange, a strong increase in the enzymatic activities were noticed (Vivancos *et al*., 2007). Ryslava *et al*. (2013) reported that infection caused by two different *Potyviruses*, *Potato virus* Y (PVY) and *Potato virus* A (PVA) resulted in the time dependent progress of NADP-ME (NADP-malic enzyme) and PEPC (Phosphoenolpyruvate carboxylase) activities.

2.2.3.5. Electrophoretic Analysis of Proteins using SDS-PAGE

Gonsalves and Ishi (1980) reported that, protein obtained by SDS-degradation of purified PRSV showed 1, 2 and 3 zones (36, 31 and 26 kDa) when analysed by PAGE. Largest protein predominated in the freshly purified preparations and smaller species in the older preparations. Roy *et al.* (1999) reported that virus particles in both PRSV-P and PRSV-W isolates migrated as a single polypeptide of apparent MW of 34 kDa. Kunkalikar *et al*. (2006); Tripathi *et al.* (2008) using SDS-PAGE estimated that, the PRSV CP, had a MW of about 36 kDa.

Siriwan *et al.* (2013) compared the protein profiles of PRSV infected and healthy papaya leaves by two dimensional PAGE. Among the observed 490 protein spots, 227 were identified using matrix-assisted laser desorption and liquid chromatography mass spectrometry. Protein profiles of BBrMV infection showed 2

major protein bands with MW's of 38 kDa and 29 kDa and 1 minor protein band of 22 kDa (Aliya, 2014).

2.2.3.6. Electrophoretic Analysis of Isozyme

Gradient PAGE electrophoresis revealed that there were different isozyme patterns in PRSV resistant and susceptible cultivars. All the papaya leaves had the same PPO with estimated MW of 53 kDa (Shaw *et al*., 1994). Valencia *et al*. (2001) used six isozyme systems, namely malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH), PO, esterase (EST), glutamate oxaloacetate transaminase (GOT) and phospoglucomutase (PGM) for isozyme analysis of PRSV infected papaya plants.

Dhanya *et al.* (2006) reported that PPO activity in healthy as well as BBrMV infected plants produced a single band with Rm value of 0.65. However in BBrMV infected sample the expression of enzyme was more prominent compared to healthy sample. They also reported another isoform of PPO with Rm value of 0.63 in infected sample which was absent in healthy samples. PO activity in BBrMV infection showed the presence of a definite band with Rm value of 0.72 (Aliya, 2014).

2.2.4. BIOLOGICAL DETECTION

Perera *et al*. (1998) reported that *Chenopodium amaranticolor* and *Chenopodium quinoa* developed local lesions whereas *Cucumis melo* gave mosaic symptoms upon PRSV infection. Roy *et al.* (1999) opined that PRSV-P and PRSV-W isolates did not produce local lesions on *Chenopodium amaranticolor* and *Chenopodium quinoa.* Out of ten plant species studied for PRSV, *Chenopodium quinoa* was shown as the only potential indicator plant and 50 per cent of the plants gave positive value in the indirect ELISA test (Kelaniyangoda and Madhubashin, 2010).

Bayot *et al.* (1990); Natsuaki (2011) reported that on mechanical inoculation with PRSV, chlorotic lesions developed on *Chenopodium quinoa.*

2.2.5. IMMUNOLOGICAL STUDIES

2.2.5.1. Enzyme linked immunosorbent assay (ELISA)

Gonsalves and Ishi (1980) detected the presence of Hawaiian isolate of PRSV using ELISA. The relative infectivity of the virus was found highest 21-27 days after inoculation. Sensitivity of ELISA increased markedly when high molarity buffer (>0.2 M Potassium phosphate buffer) or EDTA was used to extract PRSV from tissues. Bayot *et al.* (1990) detected PRSV in papaya seeds and seedlings using PAbs and MAbs. Roy *et al.* (1999) reported that PRSV-P and PRSV-W had similar serological features as determined by DAC-ELISA with polyclonal antisera, showing absorbance value between 0.34 to 1.7. Antiserum against PRSV-P also reacted with heterologous viruses such as *Alfalfa mosaic virus* (ALMV), *Egg-plant mottle virus* (EMoV), *Henbane mosaic virus* (HMV) and PVY.

Valencia *et al*. (2001) used PTA-ELISA to detect PRSV infection, using antiserum PRSV-P338. Pacheco *et al.* (2003) based on absorbance values of the PTA-ELISA, reported that, concentrations of the mild strains of PRSV were lower than the concentration of the severe strains. Kunkalikar *et al*. (2006) reported that ELISA for skin of diseased papaya, fruit and stem was mild positive but was negative for flowers, latex, seeds and roots from infected plants. Cruz *et al*. (2009) detected PRSV by direct ELISA using AGDIA kit and indirect ELISA using PAb. Singh and Shukla (2012) detected the PRSV isolate from middle gangetic plains of India, by DAC-ELISA using polyclonal antiserum against PRSV (with a dilution of 1:1000).

In Assam, DAS-ELISA was used to detect PRSV in the infected samples, results showed that of the 426 samples, 240 samples (56.3 per cent) were detected positive. Also, the mean PRSV titre values for all the positive samples were maximum in Goalpara district (1.317 nm) and minimum in Nagaon district (0.118 nm) (Talukdar *et al*., 2013). A titre of 1:50 dilution of PRSV antigen, 1:16000 of primary antibody and 1:2000 of secondary antibody was standardised for the direct plate ELISA and 1:10 dilution of antigen, 1:16000 of primary antibody and 1:2000 of

secondary antibody was standardised for PRSV detection, in direct plate ELISA with an optical density of 1.134 and 1.016 at 405 nm respectively (Muske *et al*., 2014).

2.2.5.2. Dot immunobinding assay (DIBA)

Lima *et al.* (1997) used DIBA for the detection of natural incidence of PRSV-W and WMV-2 in *Luffa operculata*. Moura *et al.* (2001) used DIBA to detect PRSV incidence in cucurbitaceous crops. DIBA was performed, with PRSV antiserum at a dilution of 1:1000, to confirm PRSV infection on sesamum (Singh *et al*., 2006). Byadagi (2008) standardised DIBA at 1:10 dilution of antigen with 1:100 dilutions of antisera for PRSV confirmation.

Thirteen of seventeen viruses tested, including PRSV-P and PRSV-W were detected by DIBA in all 10 states, from a total of 715 samples (26 cantaloupe, 6 cucumber, 46 pumpkin, 20 squash, 493 watermelon, 50 other crops and 74 weeds) from fields during 2010 and 2011 growing seasons (Ali *et al.,* 2012). Singh and Shukla (2012) detected the PRSV isolate from middle gangetic plains of India by DIBA using polyclonal antiserum for PRSV (with a dilution of 1:1000). A titre of 1:50 of PRSV antigen, 1:16000 of primary antibody and 1:2000 of secondary antibody was standardised for direct and indirect DIBA (Muske *et al*., 2014).

2.2.6. MOLECULAR DIAGNOSIS AND CHARACTERISATION

2.2.6.1. Reverse transcription-Polymerase chain reaction (RT-PCR)

A coat protein gene fragment of PRSV 950 bp was amplified by RT-PCR (Quemeda *et al.,* 1990; Wang and Yeh, 1992; Verma *et al*., 2014).

Bateson *et al*. (1994) used synthetic primers, MB11 and MB12, homologous to part of the PRSV-W (Aust-DB1) coat protein gene sequence, to amplify the coat protein gene of two Australian field isolates of PRSV-W and seven isolates of PRSV-P, including four Australian and three Asian isolates. Ha *et al*. (2008) used two pairs of degenerate primers designed from the sequences within the potyviral CI (CIForCIRev) and HC-Pro-coding regions (HPFo or HPRev), and these were shown to be highly specific to members of the genus *Potyvirus.*

Sharma *et al*. (2005); Kunkalikar *et al.* (2006); Dhanam *et al.* (2011) Siriwan *et al.* (2013) used RT-PCR, which yielded an amplicon of 850 and 550 bp respectively, corresponding to the coat protein gene of PRSV.

RT-PCR detection using the novel primers, Nlb2F and Nlb3R was proved a routine diagnostic assay for detection of all the major groups within the genus *Potyirus* (Zheng *et al*., 2010). A novel method, referred to as Peptide mass Finger Print analysis was developed for the detection of *Potyviruses* (Luo and Hao, 2012).

Usharani *et al*. (2012) used multiplex PCR to simultaneously detect PRSV and *Papaya leaf curl virus* from papaya. Tuo *et al*. (2014) used multiplex RT-PCR, with a mixture of three specific primer pairs to amplify three distinct fragments of 613 bp from the P3 gene of PRSV, 355 bp from the CP gene of PLDMV, and 205 bp from the CP gene of *Papaya mosaic virus* (PapMV).

2.2.6.2. Characterisation

Quemeda *et al.* (1990) compared nucleotide sequences of PRSV-P and PRSV-W strains, which revealed that, they share a 98.2 and 97.7 per cent identity in their NIb gene regions and coat protein genes respectively. Thus the sequences of these two strains were distinct from other *Potyvirus* types, confirming their classification as two strains of the same virus.

By direct sequencing of viral RNA, the complete genomic RNA of PRSV was determined as 10326 nucleotides in length, excluding the poly (A) tract, and contains one large open reading frame that starts at nucleotide positions 86 to 88 and ends at positions 10118 to 10120, encoding a poly protein of 3344 amino acids (Yeh *et al.,* 1992).

SilvaRosales *et al.* (2000) reported that Mexican isolates of PRSV had higher similarity to isolates from Australia and the United States than to Asian isolates. A region of about one hundred nucleotides neighbouring the putative aphid transmission triplet of the coat protein, contained repeats of an EK (glutamic acid-lysine) motif in all the sequences. Lima *et al.* (2001) reported an average degree of homology of 97.3 per cent at the nucleotide sequence among the Brazilian isolates.

Bateson *et al.* (2002) determined the coat protein sequences of PRSV biotypes (W and P) from Vietnam, Thailand, India and Philippines, and analysed them together with 28 PRSV sequences already published. In Thailand, variation was greater among PRSV-W isolates (mean nucleotide divergence 7.6 per cent) than PRSV-P isolates (mean 2.6 per cent), but in Vietnamese populations the P and W biotypes were more but similarly diverse. Phylogenetic analyses of PRSV with its closest known relative, *Moroccan watermelon mosaic virus* (MWMV), indicated that PRSV may have originated in Asia, particularly in the Indian subcontinent.

The complete nucleotide sequence of PRSV type P, Thai isolate (PRSVThP) was determined. The viral genome was 10323 nucleotides long and contained an open reading frame encoding a polyprotein of 3343 amino acids, flanked with 5' and 3' non coding regions of 85 and 206 nucleotides, respectively. Among ten putative proteins, P1 protein was the most variable (73.9 per cent similarity) when compared to the other full PRSV sequences, while CI protein was the most conserved protein (99.1 per cent similarity). Sequence similarity among the type P and type W isolates also suggested that the P type arose locally from type W (Attasart *et al*., 2002).

A comparison of the amino acid sequence (CP) of the South Indian strain (INP-UAS) of PRSV-P with other PRSV isolates showed that the N-termini was variable, which suggested the distinctiveness of INP-UAS linked to its geographical location (Hema and Prasad, 2004). The studies on the CP sequences of eleven PRSV isolates originating from different locations in India revealed that virus isolates from India exhibited considerable heterogeneity in the CP sequences and CP coding region varied in size from 840 to 858 nucleotides, encoding protein of 280 to 286 amino acids, with divergence up to 11 per cent (Jain *et al.*, 2004). Kunkliker and Byadgi (2004) reported that the PRSV isolate type-P from India showed the 715 bp long nucleotide sequence.

Maoka and Hataya (2005) reported that the viral RNA genome of strain LDM, synonymous to PRSV was comprised of 10,153 nucleotides, excluding the poly(A) tail, and contained one long open reading frame encoding a polyprotein of 3,269

amino acids (MW of 37.32 kDa). Bag *et al.* (2007) reported that PRSV isolates from central, eastern, northern, southern and western India were highly heterogeneous in CP length (275-289 amino acids) and amino acid sequences (up to 23 per cent). Among all the isolates, KA4, INU-01 and AP2 from southern India were unique. Maximum heterogeneity was observed in southern isolates (up to 23 per cent), followed by central (up to 11 per cent), eastern and northern (up to 10 per cent) and western (up to 7 per cent) isolates.

Byadagi (2008) reported a conserved region of 534 bp in the coat protein gene of PRSV. Comparative sequence analysis revealed that the PRSV isolates originating from India were divergent up to 11 per cent. Nucleotide sequences of the CP gene from Indian isolates of the virus had the highest levels of diversity, which indicated an ancestral Indian origin (Castillo *et al.,* 2011). Srinivasulu and Saigopal (2011) revealed greater sequence divergence, up to 18.4 and 15 per cent at nucleotide and amino acid levels, respectively within the Indian PRSV populations. All South Indian isolates were clearly separated from other geographical isolates and formed a major group in phylogenetic tree.

Omar *et al*. (2011) reported that Egyptian isolates of PRSV grouped together in a distinct clade. Comparison with PRSV sequences retrieved from Gen Bank presented nucleotide identities in the range of 87.5 to 97.1 per cent and close relationships of the Egyptian isolates with the two Venezuelan isolates and to the Mexican and USA isolates. Akthar *et al*. (2013) reported that the isolates from Bangladesh shared 86 to 95 per cent amino acid sequence identity with those reported from rest 21 of the Asia and 83 to 93 per cent amino acid sequence identity with isolates from the other parts of the world.

Materials and Methods

3. MATERIALS AND METHODS

3.1. SYMPTOMATOLOGY

Varieties of cowpea, Vellayani Jyothika (yard long bean) from Department of Olericulture, College of Agriculture, Vellayani and CO6 (bush cowpea) from TNAU were selected for the study. Similarly papaya varieties, Pusa Nanha from Pusa, New Delhi and a susceptible local variety from Instructional Farm, College of Agriculture, Vellayani were selected for the present study. Seeds were sown in pots containing potting mixture of sand, soil and cow dung in the ratio of 1:1:1. Leaves showing the typical virus symptoms were collected from the field and the culture was maintained by repeated transfers on the selected cultivars in insect proof glasshouse condition. Repeated transfers were done through mechanical inoculation using 0.1 *M* Sodium phosphate buffer (pH 7.0) (Appendix I). Symptomatology was studied by observing the development of symptoms in naturally infected as well as artificially inoculated conditions.

3.2. TRANSMISSION

3.2.1. Mechanical Transmission

Virus infected cowpea and papaya plants maintained in the insect proof glass house were used as source of virus inoculum. Sap transmission was conducted using 0.1 *M* Sodium phosphate buffer (pH 7.0). The sap was extracted from infected young leaves showing typical symptoms. Leaf tissue was homogenized with the Phosphate buffer at the rate of 1 g per 1.5 ml of the buffer using pre-chilled mortar and pestle. The homogenate was maintained in an ice box and immediately used for inoculation. The leaves of test plants were uniformly dusted with carborundum powder prior to inoculation. Inoculation was done by gently rubbing the cowpea at primary leaf stage and papaya at six leaf stage, using fore finger, dipped in the inoculum. The surface was rinsed off after 5 min with distilled water using a wash bottle. The development of symptoms on these plants was recorded up to 60 days after inoculation (DAI).

3.2.2. Seed Transmission

Transmission studies via seeds were conducted using the seeds collected from infected and healthy cowpea and papaya plants. They were planted in pots, kept in an insect proof glass house and examined for the development of symptoms up to 60 days after sowing (DAS).

3.2.3. Insect Transmission

Insect transmission studies were conducted using aphids and mealy bugs which were associated with cowpea and papaya plants (Sylvester, 1965; Golino *et al*., 2002).

Aphids, *Aphis craccivora* Koch and *Aphis gossypii* Glover (F: Aphididae) were used to test vector role of the *Potyviruses*. Virus free stock culture of the vector was maintained in healthy cowpea plants. The aphids were starved for a period of 1 h (pre-acquisition fasting period) and were allowed to feed on young infected cowpea and papaya plants for a period of 10 min (acquisition access period). Aphids (10 per plant) were then released to 10 virus free seedlings in the glass house with an inoculation access period of 24 hours and maintained in an insect proof cage.

The transmission of PRSV was done using papaya mealy (*Paracoccus marginatus* Williams and Garnara de Willink) (F:Pseudococcidae). Virus free mealy bug culture was reared on sprouted potato tubers. The reared insects were were allowed to feed on infected papaya leaves for a period of 24 h (acquisition access period). Mealy bugs (10 per plant) were then released to 10 healthy papaya seedlings, with a 1 h inoculation access period.

After inoculation access period, insects were killed using 0.1 per cent Quinalphos. The inoculated plants were isolated and kept under observation for the development of symptoms

3.3 HOST PATHOGEN INTERACTION STUDIES

Host pathogen interaction studies of healthy and diseased cowpea and papaya plants were carried out. Cowpea and papaya plants were mechanically

inoculated at primary and six leaf stage respectively and samples were taken at intervals of five, ten, fifteen, thirty and sixty days after mechanical inoculation. The analysis were carried out using varieties resistant and susceptible to mosaic and ring spot disease in cowpea and papaya respectively. The resistant variety, CO6 from TNAU and susceptible variety, Vellayani Jyothika from Department of Olericulture, College of Agriculture, Vellayani were taken for the study of viral disease in cowpea. The resistant variety, Pusa Nanha from Pusa, New Delhi and a susceptible local variety from Instructional Farm, College of Agriculture, Vellayani were taken for the study of ring spot disease in papaya.

Biochemical analysis were conducted to estimate the changes in total carbohydrates, chlorophyll, phenol and protein. Analysis of defense related enzymes such as peroxidase, polyphenol oxidase and phenylalanine ammonialyase were also done. Protein profile study was conducted using SDS-PAGE. Isozyme analysis of healthy and diseased plants was performed using native PAGE.

3.3.1. Estimation of Total Carbohydrates

Total carbohydrate content of healthy and diseased samples at various intervals after inoculation was estimated by Anthrone method (Hedge and Hofreiter, 1962). Samples of 100 mg each were weighed out and hydrolyzed using 5 ml of 2.5 *N* Hydrochloric acid (HCl) in a boiling water bath for 3 h. The hydrolysate was then neutralized with solid Sodium carbonate until the effervescence ceased. The volume was made up to 100 ml and centrifuged at 5000 rpm for 15 min. From the supernatant, 0.5 ml aliquot was taken and made up to 1 ml by adding distilled water. To this, 4 ml anthrone reagent (Appendix II) was added and heated for 8 min in a boiling water bath. This was cooled rapidly and absorbance was measured at 630 nm in a spectrophotometer (Systronics UV- VIS Spectrophotometer 118). Glucose was used as the carbohydrate standard. Carbohydrate content was expressed as mg of glucose equivalent per g of leaf tissue on fresh weight basis.

3.3.2. Estimation of Chlorophyll

Chlorophyll was estimated by the method described by Arnon (1949). Leaf sample (1 g) was finely cut and ground in a mortar with 20 ml of 80 per cent acetone. The homogenate was centrifuged at 5000 rpm for 5 min and the supernatant was transferred to a 100 ml volumetric flask. The above procedure was continued till the residue became colourless. The final volume in volumetric flask was made up to 100 ml with 80 per cent acetone. Absorbance of the solution at 645 and 663 nm was read in a spectrophotometer against the solvent (80 per cent acetone) as blank. The chlorophyll content was calculated using the following equations and expressed as mg of chlorophyll per g of leaf tissue on fresh weight basis.

$$\text{Chlorophyll a} = 12.7\ (A_{663}) - 2.69\ (A_{645}) \times \frac{V}{1000 \times V}$$

$$\text{Chlorophyll b} = 22.9\ (A_{645}) - 4.68\ (A_{663}) \times \frac{V}{1000 \times V}$$

$$\text{Total chlorophyll} = 20.2\ (A_{645}) + 8.02\ (A_{663}) \times \frac{V}{1000 \times V}$$

where A: absorbance at specific wavelengths; V: final volume of chlorophyll extract in 80 per cent acetone; W: fresh weight of tissue extracted.

3.3.3. Estimation of Total Soluble Protein

Total soluble protein content was estimated as per the procedure described by Bradford (1976). Leaf sample (1 g) was homogenized in 10 ml of 0.1 *M* Sodium acetate buffer (pH 4.7) (Appendix III) and centrifuged at 5000 rpm for 15 min at 4 ^{o}C. The supernatant was saved for the estimation of soluble protein. The reaction mixture consisted of 0.5 ml enzyme extract, 0.5 ml distilled water and 5 ml of diluted (5 times) dye solution (Appendix III). The absorbance was read at 595 nm in a spectrophotometer against reagent blank. Bovine serum albumin

was used as the protein standard. The protein content was expressed as mg albumin equivalent of soluble protein per gm on fresh weight basis.

3.3.4. Estimation of Phenol

The phenol content was estimated following the procedure described by Bray and Thorpe (1954). Leaf sample (1 g) was homogenised in 10 ml of 80 per cent ethanol. The homogenate was then centrifuged at 10000 rpm for 20 min, supernatant was saved, residue was extracted with 5 times the volume of 80 per cent ethanol and centrifuged as above. The supernatant was saved and evaporated to dryness in a boiling water bath. The residue was dissolved in 5 ml of distilled water. An aliquot of 0.2 ml was pipetted out and made up to 3 ml with distilled water. Folin- Ciocalteau reagent (0.5 ml) was added and 2 ml of 20 per cent Sodium carbonate solution was added to each tube after 3 min. This was mixed thoroughly and kept in boiling water for 1 min. The reaction mixture was cooled and absorbance was measured at 650 nm against a reagent blank. Standard curve was prepared using different concentrations of catechol. Phenol content was expressed as mg catechol equivalent of enzyme activity per g leaf tissue on fresh weight basis.

3.3.5. Estimation of Defense Related Enzymes

3.3.5.1. Estimation of Peroxidase (PO)

PO activity was assayed by spectrophotometric method as described by Srivastava (1987). Leaf sample (1 g) was homogenized in 5 ml of 0.1 M Sodium phosphate buffer (pH 6.5) (Appendix IV) to which a pinch of Polyvinyl pyrrolidone (PVP) was added. The homogenization was done at 40C using a pre-chilled mortar and pestle. The homogenate was centrifuged at 5000 rpm for 15 min at 40C. The supernatant was used as the enzyme extract for the assay of PO activity.

The reaction mixture consisting of 1 ml of 0.05 M pyrogallol and 50 µl of enzyme extract was taken in both reference and sample cuvettes, was kept in a spectrophotometer (Systronics UV- VIS spectrophotometer 118)

and the reading was adjusted to 0 at 420 nm. To initiate the reaction, 1 ml of 1 per cent hydrogen peroxide was added to the sample cuvettes and the changes in absorbance were recorded at 30 sec interval up to 180 sec. The PO activity was expressed as changes in absorbance per min per g fresh weight of tissue.

3.3.5.2. Estimation of Polyphenol oxidase (PPO)

PPO activity was determined as per the procedure given by Mayer et al. (1965). The enzyme extract was prepared as per the procedure given for the estimation of peroxidase.

The reaction mixture contained 1 ml of 0.1 M Sodium phosphate buffer (pH 6.5) and 50 µl of enzyme extract. The reaction was initiated after adding 1 ml of 0.01 M catechol. The observations were recorded in a spectrophotometer (Systronics UV-VIS spectrophotometer 118). The changes in absorbance was recorded at 495 nm at 30 sec interval up to 180 sec. PPO activity was expressed as change in the absorbance of the reaction mixture per min per g on fresh weight basis.

3.3.5.3. Estimation of Phenylalanine ammonialyase (PAL)

PAL activity was assayed spectrophotometrically by assaying the rate of conversion of L- phenyl alanine to trans- cinnamic acid at 290 nm as described by Dickerson et al. (1984). The enzyme extract was prepared by homogenizing 1 g leaf sample in 5 ml of 0.1 M Sodium borate buffer (pH 8.8) (Appendix IV) containing a pinch of PVP using chilled mortar and pestle. The homogenate was centrifuged at 10000 rpm for 10 minutes at 4 0C. The supernatant was used for the assay of PAL activity. The reaction mixture contained 3 ml of 0.1 M Sodium borate buffer (pH 8.8), 0.2 ml of enzyme extract and 0.1 ml of 12 mM L-Phenyl alanine prepared in the same buffer. The blank contained 3 ml of 0.1 M Sodium borate buffer (pH 8.8) and 0.2 ml enzyme extract. The reaction mixture and blank were incubated for 30 min at 40 0C and reaction was stopped by adding 0.2 ml of

N Hydrochloric acid (HCl). The absorbance was read at 290 nm in a spectrophotometer (Systronics UV-VIS spectrophotometer 118). PAL activity was expressed as µg of cinnamic acid produced per min per g on fresh weight basis.

3.3.6. Electrophoretic analysis of Proteins Using SDS-PAGE

Electrophoretic separation of soluble protein of cowpea and papaya leaves were carried out as per the procedure described by Laemelli (1970).

3.3.6.1. Procedure

Healthy and infected leaf samples (1 g each) were homogenized in 1 ml of cold denaturing solution at 4 °C (Appendix V). The extract was centrifuged at 5000 rpm for 15 min. The supernatant was mixed with chilled acetone in the ratio 1:1 and the protein was allowed to precipitate by keeping the mixture for 30 min at 4 °C. The sample was centrifuged at 5000 rpm for 15 min at 4°C. The supernatant was removed and the pellet was resuspended in 50 µl of denaturing solution and vortexed. The homogenate was centrifuged at 5000 rpm for 15 min. The supernatant was mixed with 10 µl of sample buffer and kept in a boiling water bath for 3 min. These samples were used for PAGE. The protein concentration was adjusted in each sample to strength of 100 µg of protein following Bradford method. Standard was prepared using known 10 µl of molecular weight marker (Protein Molecular Weight Marker, GeNei Cat No. 623110275001730) added to 10 µl sample buffer.

Separating gel was first casted followed by stacking gel by mixing the various solutions as indicated in the Appendix V. The water layered over the separating gel was removed and washed with a little electrode buffer and then the stacking gel was poured over the polymerized separating gel, after keeping the comb in position. After polymerization, the comb was removed and the samples were loaded into the wells. Standard with known molecular weight is also loaded in to one of the wells. The electrophoresis was performed at 100 V till the dye reached the separating gel. Then the voltage was increased to 200 V and

continued till the dye reached the bottom of the gel. Immediately after electrophoresis, the gel was removed from the glass plates and incubated in the staining solution for overnight with uniform shaking and later transferred to destaining solution. The protein appeared as bands in the gel and was photographed.

3.3.7. Electrophoretic analysis of Isozyme

Electrophoresis of protein extracts from plant tissues using different kinds of support media and buffer systems (Appendix VI) allows separation of the multiple forms of enzymes (isozymes) on the basis of charge and molecular size.

The present work was undertaken to study the enzyme alterations in healthy as well as virus infected cowpea and papaya leaves. Discontinuous anionic Polyacrylamide Gel Electrophoresis was conducted under non-dissociating conditions as described by Wagih and Coutts (1982) with slight modification.

3.3.7.1. Procedure

Soluble and ionically bound enzymes were extracted by grinding the sample under chilled condition in 50 m*M* Tris-HCl (pH 7.6) in the ratio of 1:1 w/v. The homogenate was centrifuged at 15,000 rpm for 10 min at 4 °C. The resulting supernatant was used for isozyme analysis. The protein content was adjusted in each sample to the strength of 100 µg of protein following Bradford method. Proteins extracted by 50 m*M* Tris-HCl (pH 7.6) were separated by gel electrophoresis in 7.5 per cent gel. The gel was prepared using the stock solution prepared for protein gel electrophoresis without SDS (native gel). Triton X-100 (2%) was added in the place of SDS. The gel was incubated in 0.6 *M* Sodium acetate buffer (pH 5.4) containing 0.5 per cent O-dianisidine HCl for 30 min at room temperature. The gel was transferred to 0.1 *M* Hydrogen peroxide until visible bands were developed.

3.4. IMMUNOLOGICAL STUDIES

The virus isolates from different diseased samples were diagnosed using monoclonal and polyclonal antibodies developed specifically against *Potyviruses*

infecting cowpea (*Cowpea aphid-borne mosaic virus and Blackeye cowpea mosaic virus*) and papaya (*Papaya ring spot virus)* using Enzyme linked immunosorbent assay (ELISA) and Dot immunobinding Assay (DIBA).

3.4.1. Direct antigen coating-Enzyme linked immunosorbent assay (DAC-ELISA)

ELISA was carried out to detect the presence of BlCMV/CABMV and PRSV in infected cowpea and papaya leaves. The procedure described by Huguenot *et al*. (1993) was followed for the detection.

Infected young leaf (1 g) was homogenized in 5 ml of coating buffer (Carbonate buffer) containing 2 per cent (w/v) PVP under chilled condition. Healthy plant extract was prepared by using leaves of healthy plants.

The homogenate was centrifuged at 5000 rpm for 10 min at 4 °C. Samples were dispensed at the rate of 100 µl into Nunc immunological plates. The treatments were replicated thrice. After incubation for 2h at 37 °C the wells were washed with Phosphate Buffer Saline-Tween (PBS-T) three times each for duration of 3 min using an ELISA plate washer (PW-40, BIORAD). Blocking was done with 100 µl of 5 per cent spray dried milk (SDM) for 2 h at 37 °C. After incubation plates were washed with PBS-T as before. Then plates were treated with 100 µl of monoclonal/polyclonal antibodies, diluted in PBS-T Polyvinyl pyrrolidone ovalbumin (PBS-TPO). Three replications were maintained for each treatment and incubated overnight at 4 °C. The plates were washed again with PBS-T and then treated with 100 µl of anti-mouse immunoglobulin/goat-antirabbit immunoglobulin (SIGMA-Aldrich) diluted in PBS-TPO and incubated for 2 h at 37 °C. Wells were washed with PBS-T as before. The substrate p-nitro phenyl phosphate in diethanolamine buffer (1 mg per ml) was added to each well (100 µl per well) and incubated for 1 h at 37 °C. Reaction was stopped by adding 50 µl of 4 per cent Sodium hydroxide. The absorbance was read at 405 nm in an ELISA reader (Microplate Reader 680, BIORAD) (Appendix VII).

Monoclonal antibodies (MAbs) against BlCMV (15E6 and 16G5) and CABMV (6C12, 7D9, 12S9, 5H5, 6C10, IF5, 7A10, 5D17 and 9B8) and polyclonal antibody (PAb) against BlCMV (H2) and CABMV (H4) at 1:200 dilutions in PBS-TPO were used for detection. Also polyclonal antibody against PRSV and *Potyvirus* group antiserum at 1:500 dilution were used for detection. For MAbs, alkaline phosphatase conjugated anti-mouse immunoglobulin (SIGMA-Aldrich) and for PAbs, alkaline phosphatase conjugated goat-antirabbit immunoglobulin (SIGMA-Aldrich) diluted in PBS-TPO (1:10,000 v/v) was used as the secondary conjugate.

3.4.1.1. Detection of presence of virus in mealy bug using DAC-ELISA

The mealy bugs reared on sprouted potato tubers were given an acquisition access period of 48 h with mosaic infected papaya leaves. These insects were assayed for the presence of PRSV by DAC-ELISA. Fifty such adult insects were ground separately in 1 ml of Phosphate buffer. DAC-ELISA was performed as per the procedure described in para 3.4.1. Polyclonal antibody against PRSV and *Potyvirus* group antibody were used as the antiserum. The absorbance was read at 405 nm in an ELISA reader (BIORAD, Model 680 Microplate reader).

3.4.2 Dot immunobinding assay (DIBA)

DIBA was carried out to detect the presence of BlCMV/CABMV and PRSV in infected cowpea and papaya leaves. Polyclonal antibodies against BlCMV (H2), CABMV (H4) and PRSV were used for the study.

3.4.2.1. Procedure

Tissue was extracted in antigen extraction buffer (1:10 w/v) and filtered through cheese cloth. Expressed sap (0.8 ml) was taken in an eppendorf tube to which 0.4 ml chloroform was added. The mixture was vortexed and centrifuged at 12,000 rpm for 2 min. The clarified sap (upper aqueous layer) was mixed with antigen extraction buffer (1:4 ratio) and vortexed. Nitrocellulose membrane (NCM) in squares of 1 x 1 cm was floated in Tris Buffer Saline (TBS) and air dried. The sample (10 µl) was spotted at the centre of each square and allowed to

dry. Treated NCM was immersed in blocking solution with gentle oscillation for 1 h at room temperature. NCM was rinsed in TBS for 10 min and incubated overnight at 4 °C in crude antiserum diluted in TBS-Spray Dried Milk (SDM). NCM was again rinsed in TBS for 10 min and incubated for 1 h at room temperature in secondary antibody (antirabbit IgG alkaline phosphatase conjugate diluted in TBS-SDM). After rinsing in TBS for 10 min, NCM was incubated in a solution of Nitro blue tetrazolium and Bromo chloro indolyl phosphate at room temperature in the dark for colour development. NCM was rinsed in fixing solution for 10 min after the colour development and then air dried between Whatman filter paper sheets and stored (Appendix VIII).

3.5. MOLECULAR DETECTION AND CHARACTERISATION

Molecular diagnosis via Reverse Transcription-Polymerase chain reaction (RT-PCR) was performed for the detection of the *Potyviruses* infecting cowpea and papaya. RNA extraction was done using Tri soln (GeNei Catalogue No. 612106481001730).

3.5.1. Trizol Method of RNA isolation

3.5.1.1. Sample preparation

Trisoln (1 ml) was added to 100 mg of liquid nitrogen ground leaf sample and centrifuged at 12,000 rpm for 10 min at 4 ^{0}C. The cleared supernatant was transferred to a new tube and incubated for 5 min at room temperature. Chloroform (200 µl per 1 ml of Trisoln) was added to the supernatant and the tube was shaken vigorously for 15 sec. Tube was incubated for 2-3 min at room temperature and centrifuged at 12,000 rpm for 15 min at 4 ^{0}C. The upper, clear aqueous phase of the sample was pipetted out for the isolation of RNA.

3.5.1.2. RNA isolation

100 per cent Isopropanol (500 µl) was added to the clear phase, mixed gently and incubated for 10 min at room temperature. The mixture was centrifuge at 12,000 rpm for 10 min at 4 ^{0}C.

3.5.1.3. RNA wash

The supernatant was removed from the tube leaving only the RNA pellet. The pellet was washed with 1 ml of 75 per cent ethanol, vortexed briefly and centrifuged at 7,500 rpm for 5 min at 4 ^{0}C. The wash was discarded and the pellet was air dried for 5 to 10 min.

3.5.1.4. RNA resuspension

The RNA pellet was resuspended in RNAse free water, followed by incubation in a waterbath (55 to 60 ^{0} C for 15 min). RNA was later stored at -70^{0} C.

3.5.1.5. Agarose Gel Electrophoresis for RNA Quality Check

The quality of RNA isolated was checked using agarose gel electrophoresis. An agarose gel of 1.2 per cent was prepared in 1X TAE buffer and ethidium bromide was added (0.5 µl per L). An aliquot of the RNA sample (3µl) mixed with loading dye was loaded. The gel was run at 5 V cm^{-1} (Hoefer Powerpack, Germany) for 30 min. The gel was then visualized using BIORAD Molecular Imager (Gel DOC ^{TM} XR+).

3.5.2. cDNA Synthesis

The RNA isolated from leaf samples reverse transcribed to cDNA using Two Step AMV RT-PCR kit (GeNei, Catalogue No. 610662400011730).

3.5.3. Primer Designing

Primer designing was based on the nucleotide sequence of the coat protein gene, availed from the National Center for Biotechnology Information (NCBI), using Primer 3 software. Annealing temperature was fixed at 55 ^{0}C, using the Quick primer test (Fast PCR software).

3.5.4. Reverse transcription-Polymerase chain reaction (RT-PCR)

RT-PCR reaction was performed using the primer pairs designed during the study.

3.5.4.1. Primers used

Primer	Sequence
BlCMV-F	5'- ATCCAAAACATCGGATCGAG-3'
BlCMV- R	5'- TTTTGGTTAACGTCCCTTGC-3'
CABMV-F	5'-GAAAACGCAAAACCCACACT-3'
CABMV-R	5'-CGGCCTTCATTTGTGCTATT-3'
PRSV-F	5'- CTCGTGCCACTCAATCTCAA -3'
PRSV-R	5'-TCAGCTTGAGTTTCCCCATC -3'

3.5.4.2. RT-PCR Analysis

RT-PCR amplification reactions were carried out in a 20 µl reaction volume which contained: 1X Phire PCR buffer (contains 1.5 m*M* $MgCl_2$), 0.2m*M* each dNTPs (dATP, dGTP, dCTP and dTTP), 1 µl cDNA, 0.2 µl Phire Hotstart II DNA polymerase enzyme, 0.1 mg per ml BSA and 3 per cent DMSO, 0.5*M* Betaine and 5p*M* of forward and reverse primers.

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

3.5.4.3. RT-PCR amplification profile

98 ^{o}C - 30 sec

98 ^{o}C - 5 sec
55 ^{o}C - 10 sec } 40 cycles
72 ^{o}C - 15 sec

72 ^{o}C - 60 sec
4 ^{o}C - ∞

3.5.4.4. Agarose Gel electrophoresis of RT-PCR products

The RT-PCR products were checked in 1.2 per cent agarose gels prepared in 0.5X TAE buffer containing 0.5 µg/ml ethidium bromide. 1 µl of 6X loading dye was mixed with 5 µl of PCR products, loaded and electrophoresis was performed at 75 V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 h. The molecular standard used was a 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad) (Appendix IX).

3.5.5. ExoSAP-IT Treatment

ExoSAP-IT (GE Healthcare) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications.

PCR product of 5µl is mixed with 2 µl of ExoSAP-IT and incubated at 37 ^{o}C for 15 min followed by enzyme inactivation at 80 ^{o}C for 15 min.

3.5.6. Sequencing using BigDye Terminator v3.1

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol.

The PCR mix consisted of the following components:

PCR Product (ExoSAP treated)	-	10-20 ng
Primer	-	3.2 p*M* (Forward or Reverse)
Sequencing Mix	-	0.28 µl
Reaction buffer	-	1.86 µl
Sterile distilled water	-	made up to 10µl

The sequencing PCR temperature profile consisted of a 1st cycle at 96 ^{o}C for 2 min followed by 30 cycles at 96 ^{o}C for 30 sec, 50 ^{o}C for 40 sec and 60 ^{o}C for

4 min.

3.5.7. Post Sequencing PCR Clean up

A master mix I of 10µl milli Q and 2 µl 125 m*M* EDTA per reaction and master mix II of 2 µl of 3 *M* sodium acetate pH 4.6 and 50 µl of ethanol were prepared. Master mix I (12 µl) was added to each reaction containing 10µl of reaction contents and was properly mixed. Master mix II (52µl) was added to each reaction. The contents were mixed by inverting and incubated at room temperature for 30 min. Further it was centrifuged at 14,000 rpm for 30 min. The supernatant was decanted and 100 µl of 70 per cent ethanol was added. It was then spinned at 14,000 rpm for 20 min. The supernatant was decanted and 100 µl of 70 per cent ethanol was added. The supernatant was decanted and pellet was air dried. The cleaned up air dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems).

3.5.8. Sequence Analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems).

3.5.9. Characterisation

The molecular detection of the *Potyviruses* was confirmed by performing a similarity search using Basic Local Alignment Search Tool (BLAST) in the National Centre for Biotechnology Information (NCBI) database and the sequences (Appendix X) were matched with existing NCBI database.

The nucleotide sequences based on the coat protein region of the *Potyvirus* isolates and other related *Potyviruses* were retrieved from NCBI Genbank data base (USA) and compared. Multiple nucleotide sequence alignment and phylogenetic analysis was done using NCBI BLAST Tree Widget. Similarly, multiple amino acid sequence alignment of the *Potyviruses* were also done using NCBI BLAST.

Comparative nucleotide sequence alignment of the two *Potyvirus* isolates was done using Clustal Omega and phylogenetic analysis through Tree

View software. Extend of similarity in the nucleotide sequences of the *Potyviruses* was obtained from the Percentage Identity Matrix (Clustal Omega).

Results

4. RESULTS

The present study on "Immunomolecular detection and characterisation of *Potyviruses* infecting cowpea and papaya" was conducted during the period of 2013-2015 at the Department of Plant Pathology, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala, to study on the symptomatology and transmission of *Potyviruses* infecting cowpea and papaya, along with their biological, immunological, molecular detection and characterisation. The results obtained from the glass house and laboratory experiments are summarized below:

4.1 *Blackeye cowpea mosaic virus* (BlCMV)

4.1.1 SYMPTOMATOLOGY

Mechanically inoculated seedlings of cowpea cultivar, Vellayani Jyothika expressed symptoms of virus infection 7 days after inoculation (DAI). Initial symptoms appeared as vein clearing on the newly emerged trifoliate leaves. Vein clearing symptoms were also noticed in seedlings (Plate 1a) raised from seeds of virus infected plants. Later dark green and light green mottling on the leaves was observed. Typical symptoms noted were mosaic (Plate 1b) and vein banding (Plate 1c), followed by reduction in leaf size and distortion. Severe stunting of plants (Plate 1e) was also noted along with malformation of flowers (Plate 1d) and reduced pod production.

Majority of the plants of the resistant cultivar CO6, failed to develop any external symptoms on mechanical inoculation and remained apparently healthy. However, a very few plants developed mild vein clearing symptoms, 10-12 DAI, but the disease severity rapidly declined and plants retained their apparent healthy appearance.

The seed borne nature of BlCMV, along with the high susceptibility of Vellayani Jyothika to aphids enabled easy incidence and spread of the disease under field condition. The symptoms produced on cowpea plants by mechanical inoculation resembled those observed naturally under field conditions.

Plate 1a. Vein clearing

Plate 1b. Mosaic

Plate1c. Vein banding

Symptoms of BlCMV infection in cowpea

Plate 1d. Floral deformation

Plate 1e. Stunted growth

Symptoms of BlCMV infection in cowpea

4.1.2 TRANSMISSION STUDIES

The transmission studies were conducted on susceptible cowpea cultivar Vellayani Jyothika.

4.1.2.1 Mechanical Transmission

Sap transmission of BlCMV from diseased to healthy cowpea plants at primary leaf stage was done using 0.1 *M* Sodium phosphate buffer (pH 7.0). The initial symptoms of vein clearing was noticed on the newly emerging leaves, 7 DAI and 80 per cent transmission was recorded (Table 1).

4.1.2.2 Transmission through Seeds

Transmission of BlCMV through seeds, obtained from infected cowpea plants were studied. Symptoms were observed on seedlings obtained from infected cowpea plants, even from the primary leaf stage. Seed transmission of 56 per cent was recorded in the present study (Table 2).

4.1.2.3 Insect Transmission

Insect transmission studies were carried out using two aphid species *viz*. *Aphis craccivora* Koch (Plate 2a) and *Aphis gossypii* Glover (Plate 2b) to find out the comparative efficacy of transmission. The results revealed that both the aphids effectively transmitted the virus (30 per cent transmission respectively) (Table 3).

4.1.3 HOST PATHOGEN INTERACTION STUDIES

Study was made to quantify the effects of BlCMV on the total chlorophyll and carbohydrate contents of the resistant (CO6) (Plate 3a) and susceptible (Vellayani Jyothika) (Plate 3b) genotypes of cowpea, at different stages of inoculation *viz.* at five, ten, fifteen, thirty and sixty days after mechanical inoculation. Investigations were also conducted on the quantitative estimation of phenol, protein content, peroxidase, polyphenol oxidase and phenylalanine ammonialyase activities at different stages of inoculation, to investigate their role

Table 1. Mechanical transmission studies of BlCMV

Sl. No.	Test plant	No. of plants inoculated	No. of plants infected	Per cent transmission
1	Cowpea	25	20	80

Table 2. Seed transmission studies of BlCMV

Sl. No.	Sources of seed	No. of seeds sown	No. of plants infected	Per cent transmission
1	Healthy cowpea	50	0	0
2	Diseased cowpea	50	28	56

Table 3. Insect transmission studies of BlCMV

Sl. No.	Insect species	No. of insects released per plant	No. of plants inoculated	No. of plants infected	Per cent transmission
1	*Aphis craccivora*	10	10	3	30
2	*Aphis gossypii*	10	10	3	30

Plate 2a. *Aphis gossypii* Glover

Plate 2b. *Aphis craccivora* Koch

Insect vectors used for transmission study of BlCMV

Plate 3a. CO6

Plate 3b. Vellayani Jyothika

Varieties of cowpea used for the study

in BlCMV inoculated and uninoculated plants of resistant and susceptible cowpea genotypes. The data obtained from the experiments were analysed separately for each parameter and subjected to simple CRD for the different treatments.

4.1.3.1 Estimation of Total Carbohydrate

The estimation of total carbohydrates was done as per the procedure given by Hedge and Hofreiter (1962) and the results are presented in the Table 4 and Figure 1.

In the case of CO6, carbohydrate content was found to increase from 5 DAI (15.06 mg g^{-1}) and attained a peak at 10 DAI (23.07 mg g^{-1}) and thereafter maintained a lower level. In the case of inoculated plants of Vellayani Jyothika, carbohydrate increased from 5 DAI (8.17 mg g^{-1}) to reach the maximum at 15 DAI (15.95 mg g^{-1}), and there after declined. Beyond 5 DAI, a significant decrease was noticed compared to the uninoculated plants.

4.1.3.2 Estimation of Chlorophyll

The chlorophyll contents of resistant and susceptible cultivars were estimated as per the procedure given by Arnon (1949) and the results are present in Tables 5, 6 and 7 and Figs 2, 3 and 4. The samples were estimated for chlorophyll a, chlorophyll b and total chlorophyll at different days after inoculation. The three traits showed an increasing trend as the plant matures for both inoculated and uninoculated plants of resistant and susceptible genotypes.

Chlorophyll a of the inoculated plants of resistant genotype, CO6 showed an increase from 5 DAI (1.02 mg g^{-1}) to 60 DAI (2.10 mg g^{-1}), while chlorophyll b increased from 5 DAI (0.43 mg g^{-1}) to 60 DAI (0.46 mg g^{-1}). Total chlorophyll content also increased from 5 DAI (1.45 mg g^{-1}) to 60 DAI (2.56 mg g^{-1}). In case of inoculated plants of Vellayani Jyothika, the chlorophyll a increased from 5 DAI (1.08 mg g^{-1}) to 60 DAI (1.57 mg g^{-1}), while chlorophyll b increased from 5 DAI (0.41 mg g^{-1}) to 60 DAI (0.87 mg g^{-1}). Total chlorophyll content also raised from 5 DAI (1.49 mg g^{-1}) to 60 DAI (2.44 mg g^{-1}). The results revealed that

chlorophyll a, chlorophyll b and total chlorophyll contents were on par in both inoculated and uninoculated plants of CO6 at 10, 15, 30 and 60 DAI. Chlorophyll a, chlorophyll b and total chlorophyll contents of inoculated susceptible plants decreased significantly when compared to uninoculated control plants throughout the period of observation.

4.1.3.3 Estimation of Phenol

Estimation of phenol was carried out as per the procedure given by Bray and Thorpe (1954) and results are summarised in Table 8 and Fig 5.

In the case of CO6, phenol content reached the maximum at 10 DAI (16.30 mg g^{-1}), followed by a marked reduction at 15 DAI (2.10 mg g^{-1}) and a gradual increase there after. Vellayani Jyothika on inoculation, also showed a similar trend in phenol accumulation. The maximum phenol content was observed at 10 DAI (6.47 mg g^{-1}), followed by a significant reduction at 15 DAI (2.25 mg g^{-1}). However, the phenol content remained the same at 30 and 60 DAI (4.23 mg g^{-1}). The results showed a significant increase in the phenol content of the inoculated plants of resistant genotype, compared to the control plants and susceptible genotype, except 5 DAI. Vellayani Jyothika recorded no significant difference in phenol content at 30 and 60 DAI.

4.1.3.4 Estimation of Total Soluble Protein

Estimation of total soluble protein content was carried out as per the procedure given by Bradford (1976) and results are presented in Table 9 and Fig 6.

In CO6, the initial protein content at 5 DAI (1.53 mg g^{-1}) reached a maximum at 60 DAI (6.61 mg g^{-1}). The protein content of Vellayani Jyothika, raised from 5 DAI (1.20 mg g^{-1}) and reached the maximum at 30 DAI (2.90 mg g^{-1}). There after the protein content declined at 60 DAI (2.56 mg g^{-1}). The inoculated resistant genotype, showed a significant increase in protein content, compared to the uninoculated plants and susceptible genotype, throughout the

Table 4. Changes in total carbohydrate content in response to BlCMV inoculation

Varieties	Treatments	*Total carbohydrate content (mg g^{-1} fresh weight)				
		Days after inoculation				
		5	10	15	30	60
CO 6	Uninoculated	14.66[a]	23.73[a]	14.93[c]	10.07[b]	14.23[a]
	Inoculated	15.06[a]	23.07[a]	14.96[c]	9.61[b]	14.00[a]
Vellayani Jyothika	Uninoculated	8.20[b]	19.23[b]	18.31[a]	11.20[a]	11.23[b]
	Inoculated	8.17[b]	11.17[c]	15.95[b]	6.07[c]	10.09[c]
CD (0.05)		0.618	0.926	0.709	0.563	0.244

*Mean of three replications

Table 5. Changes in chlorophyll a content in response to BlCMV inoculation

Varieties	Treatments	*Chlorophyll a content (mg g^{-1} fresh weight)				
		Days after inoculation				
		5	10	15	30	60
CO6	Uninoculated	1.14[a]	1.20[b]	1.44[a]	1.95[a]	2.13[a]
	Inoculated	1.02[c]	1.18[b]	1.42[a]	1.96[a]	2.10[a]
Vellayani Jyothika	Uninoculated	1.16[a]	1.29[a]	1.32[b]	1.52[b]	1.83[b]
	Inoculated	1.08[b]	1.18[b]	1.29[c]	1.40[c]	1.57[c]
CD (0.05)		0.037	0.029	0.034	0.042	0.179

*Mean of three replications

Table 6. Changes in chlorophyll b content in response to BlCMV inoculation

Varieties	Treatments	*Chlorophyll b content (mg g^{-1} fresh weight)				
		Days after inoculation				
		5	10	15	30	60
CO6	Uninoculated	0.42[b]	0.42[b]	0.46[b]	0.42[b]	0.46[c]
	Inoculated	0.43[ab]	0.41[b]	0.45[b]	0.42[b]	0.46[c]
Vellayani Jyothika	Uninoculated	0.45[a]	0.54[a]	0.74[a]	0.82[a]	0.91[a]
	Inoculated	0.41[b]	0.35[c]	0.40[c]	0.43[b]	0.87[b]
CD (0.05)		0.029	0.024	0.022	0.018	0.018

*Mean of three replications

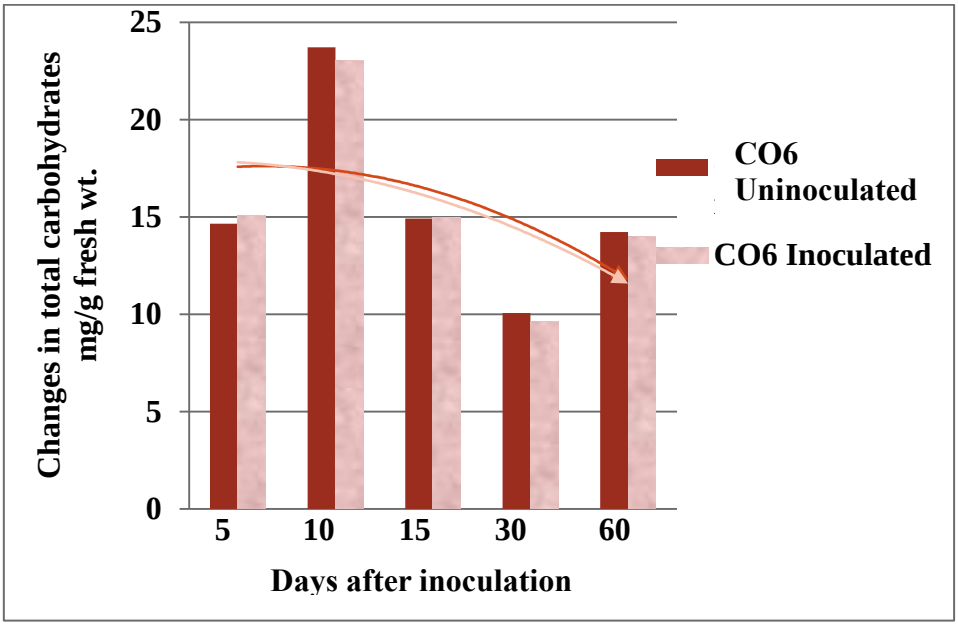

Fig 1a. CO6

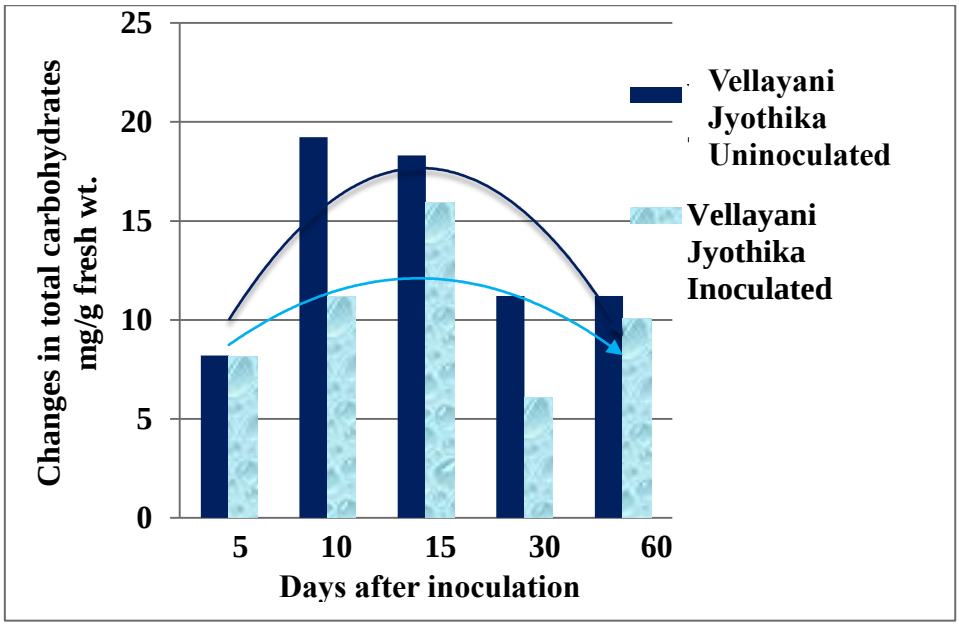

Fig 1b. Vellayani Jyothika

Changes in total carbohydrate content in response to BlCMV inoculation

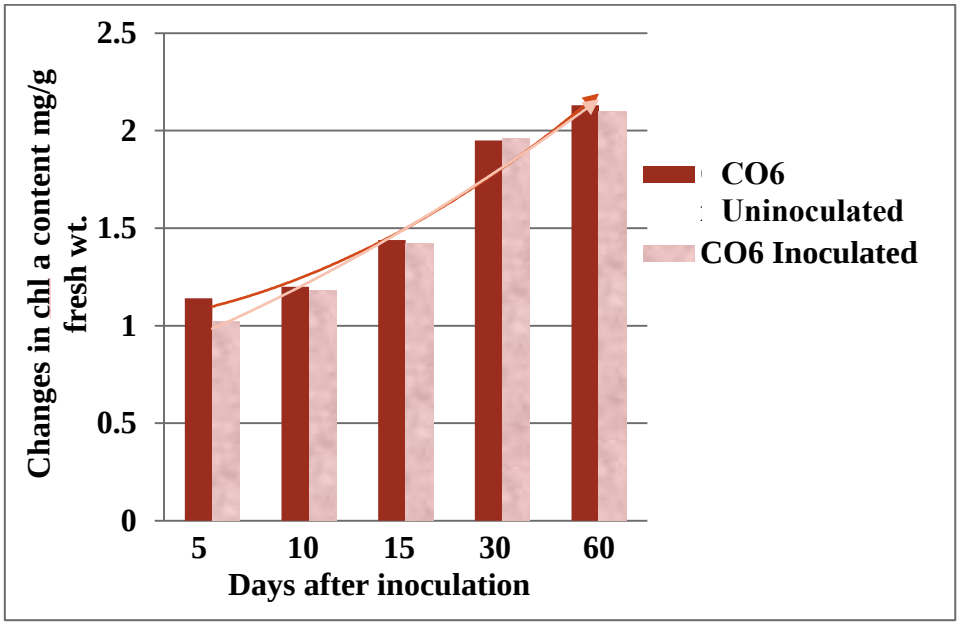

Fig 2a. CO6

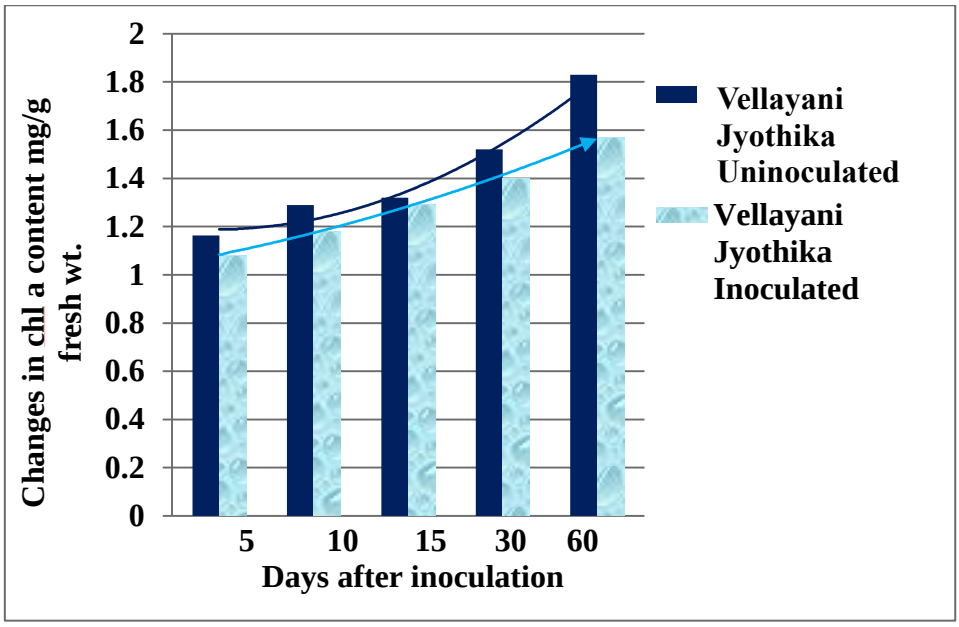

Fig 2b. Vellayani Jyothika

Changes in chlorophyll a content in response to BlCMV inoculation

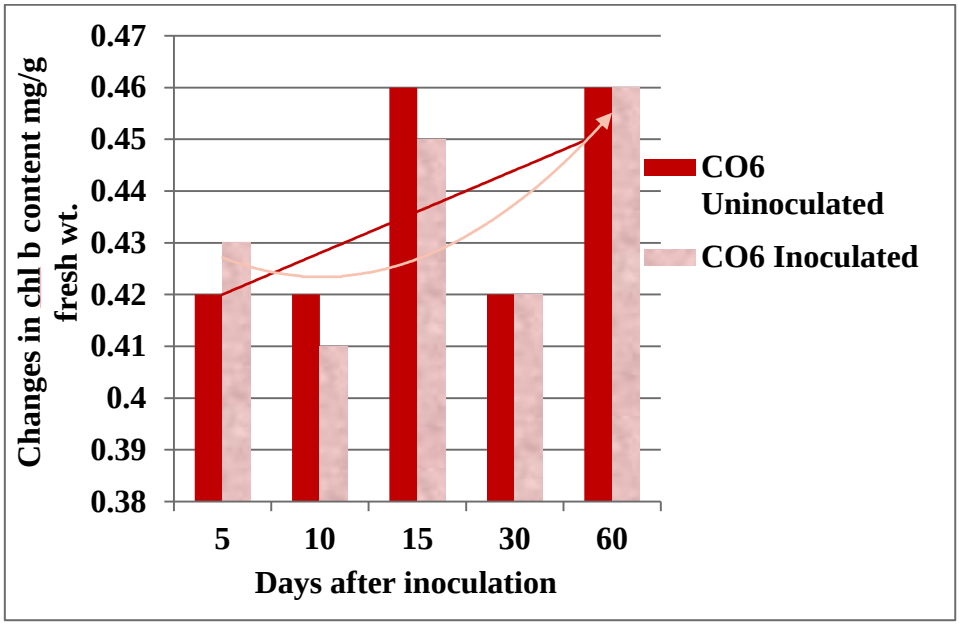

Fig 3a. C06

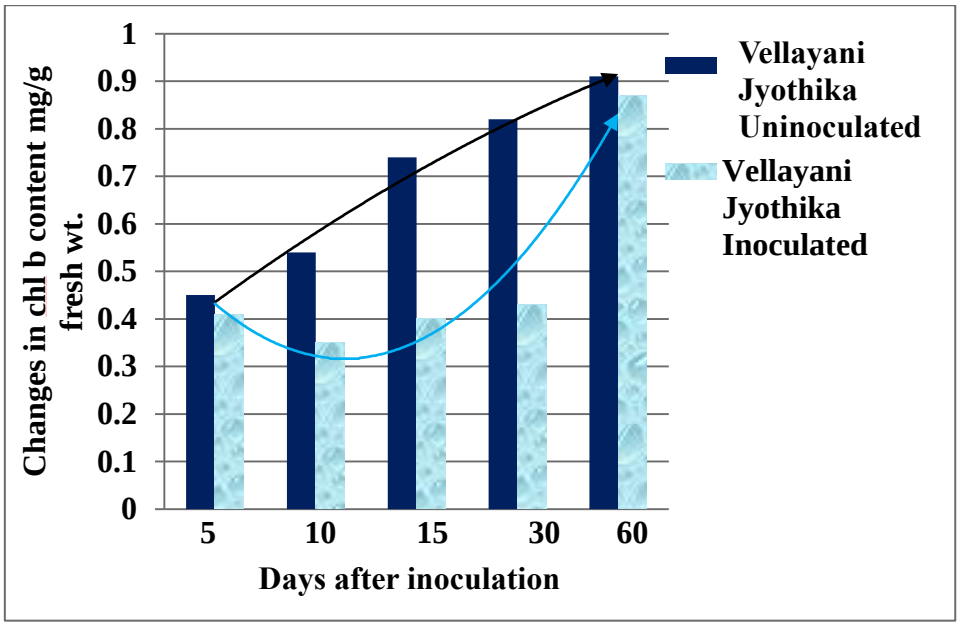

Fig 3b. Vellayani Jyothika

Changes in chlorophyll b content in response to BlCMV inoculation

Table 7. Changes in total chlorophyll content in response to BlCMV inoculation

Varieties	Treatments	*Total chlorophyll content (mg g^{-1} fresh weight)				
		Days after inoculation				
		5	10	15	30	60
CO6	Uninoculated	1.56[b]	1.61[b]	1.90[b]	2.38[a]	2.58[b]
	Inoculated	1.45[d]	1.61[b]	1.89[b]	2.37[a]	2.58[b]
Vellayani Jyothika	Uninoculated	1.61[a]	1.83[a]	2.06[a]	2.34[b]	2.74[a]
	Inoculated	1.49[c]	1.53[c]	1.70[c]	1.83[c]	2.44[c]
CD (0.05)		0.018	0.024	0.023	0.018	0.018

*Mean of three replications

Table 8. Changes in phenol content in response to BlCMV inoculation

Varieties	Treatments	*Phenol content (mg g^{-1} fresh weight)				
		Days after inoculation				
		5	10	15	30	60
CO6	Uninoculated	2.24[b]	3.82[c]	1.91[b]	5.43[b]	3.57[c]
	Inoculated	1.46[c]	16.3[a]	2.90[a]	6.17[a]	12.27[a]
Vellayani Jyothika	Uninoculated	1.55[c]	3.80[c]	1.54[c]	4.07[c]	4.10[b]
	Inoculated	3.07[a]	6.47[b]	2.25[a]	4.23[c]	4.23[b]
CD (0.05)		0.202	0.401	0.210	0.356	0.339

*Mean of three replications

Table 9. Changes in total soluble protein content in response to BlCMV inoculation

Varieties	Treatments	* Total soluble protein content (mg g^{-1} fresh weight)				
		Days after inoculation				
		5	10	15	30	60
CO6	Uninoculated	1.15[b]	2.48[b]	3.91[b]	4.06[b]	6.23[b]
	Inoculated	1.53[a]	2.53[a]	5.73[a]	4.41[a]	6.61[a]
Vellayani Jyothika	Uninoculated	1.00[c]	1.36[c]	1.66[d]	2.89[c]	2.52[c]
	Inoculated	1.20[b]	1.35[c]	2.23[c]	2.90[c]	2.56[c]
CD (0.05)		0.102	0.045	0.073	0.127	0.151

*Mean of three replications

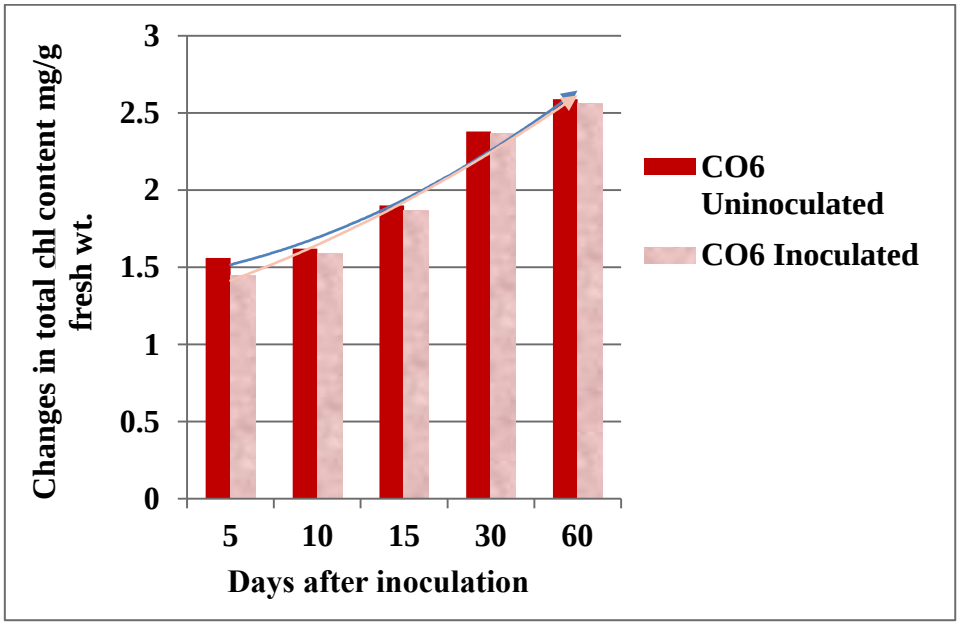

Fig 4a. CO6

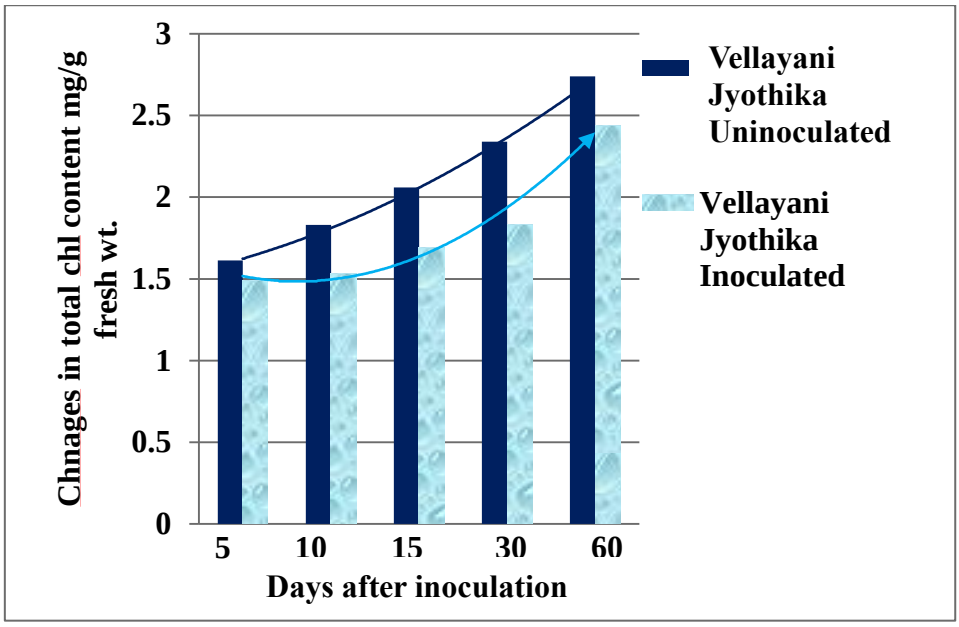

Fig 4b. Vellayani Jyothika

Changes in total chlorophyll content in response to BlCMV inoculation

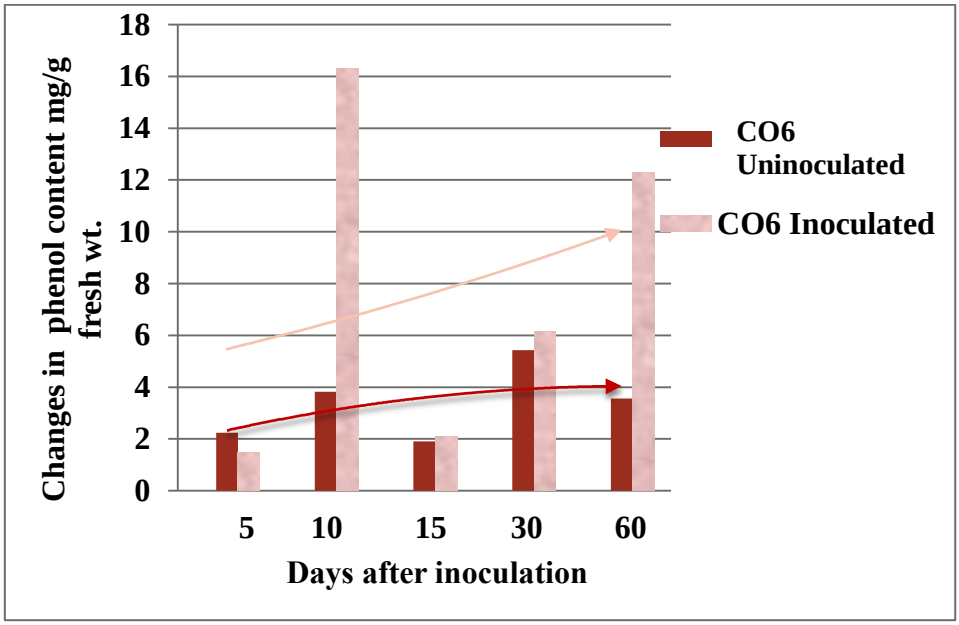

Fig 5a. CO6

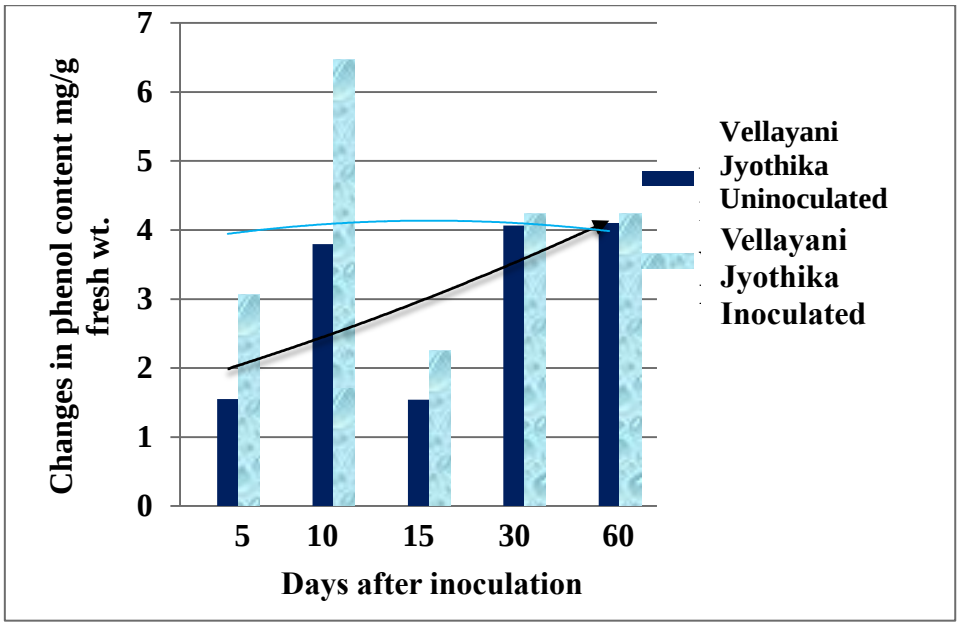

Fig 5b. Vellayani Jyothika

Changes in phenol content in response to BlCMV inoculation

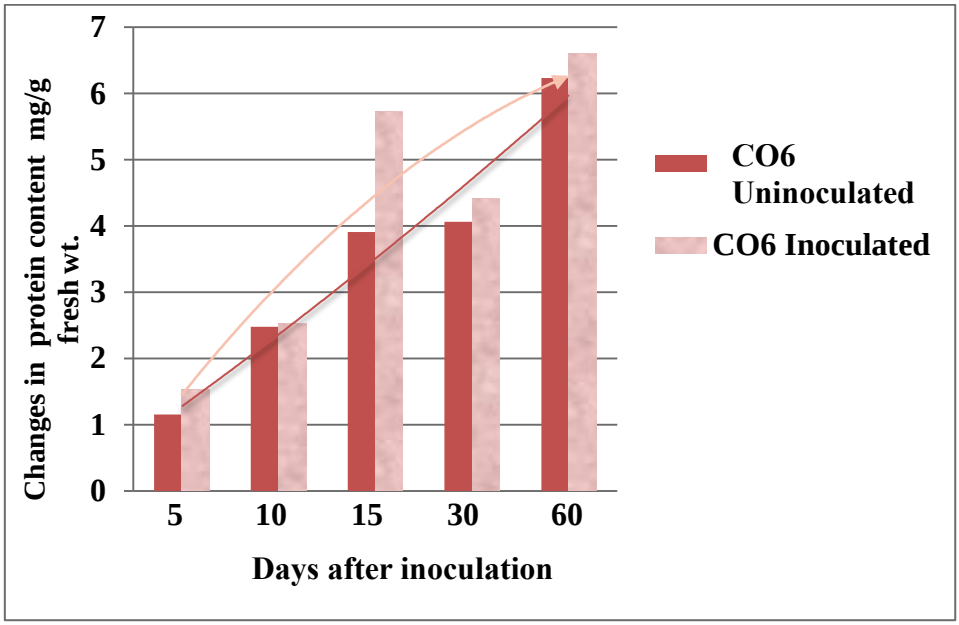

Fig 6a. CO6

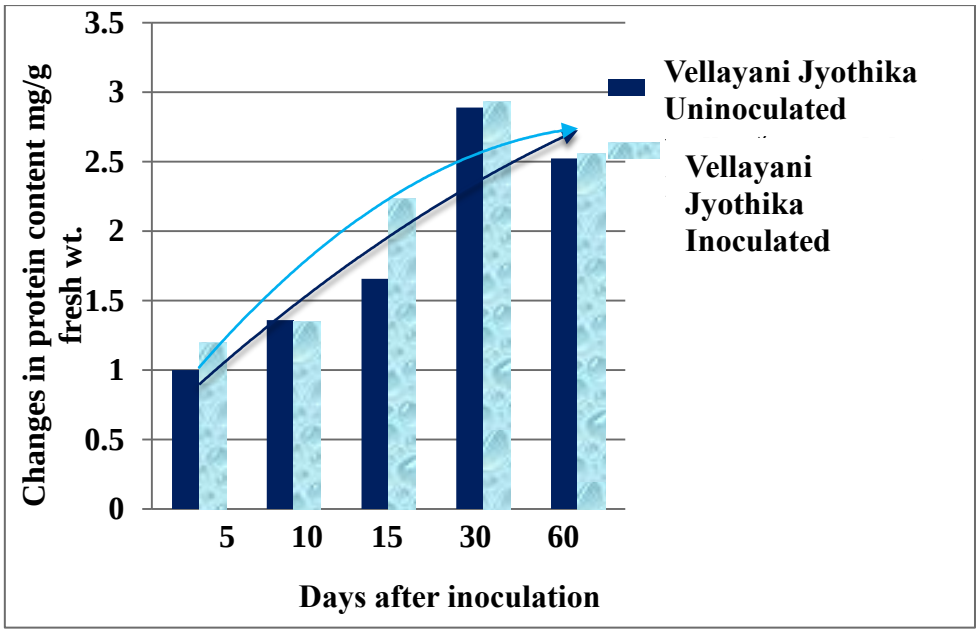

Fig 6b. Vellayani Jyothika

Changes in total soluble protein content in response to BlCMV inoculation

period of observation. Inoculated susceptible plants recorded no significant difference at 30 and 60 DAI.

4.1.3.5 Estimation of Defense Related Enzymes

4.1.3.5.1 Estimation of Peroxidase (PO)

Estimation of peroxidase activity was carried out as per the procedure given by Srivastava (1987) and results are presented in Table 10 and Fig 7.

The PO activity of CO6 was found to increase from 5 DAI (58.2 mg g^{-1}) and attained a peak at 30 DAI (81.53 mg g^{-1}) and declined there after. Vellayani Jyothika also recorded a similar trend in PO induction, activity increased from 5 DAI (9.35 mg g^{-1}) and attained the maximum activity at 30 DAI (32.10 mg g^{-1}) which declined there after. Enzyme activity was induced in the inoculated plants of both genotypes. Significant increase in PO activity was noted for the inoculated plants of resistant genotype, throughout the period of observation.

4.1.3.5.2 Estimation of Polyphenol oxidase (PPO)

Estimation of polyphenol oxidase activity was conducted as per the procedure given by Mayer *et al.* (1965) and results are given in Table 11 and Fig 8.

In CO6, the initial PPO activity at 5 DAI (1.60 min^{-1} g^{-1}) reduced to a stable value at 10 and 15 DAI (0.21 min^{-1} g^{-1}), later increased and attained the maximum activity at 60 DAI (4.27 min^{-1} g^{-1}). Vellayani Jyothika, recorded the maximum activity at 5 DAI (1.22 min^{-1} g^{-1}) which maintained a lower level throughout the period of observation. The resistant genotype, CO6 on inoculation, exhibited a significant increase in enzyme activity 5, 30 and 60 DAI. However, the susceptible genotype showed a significant increase up to 30 DAI, followed by a non-significant difference.

Table 10. Changes in peroxidase activity in response to BlCMV inoculation

Varieties	Treatments	*Peroxidase activity (changes in absorbance min^{-1} g^{-1} fresh weight)				
		Days after inoculation				
		5	10	15	30	60
CO6	Uninoculated	5.45[c]	2.50[c]	3.14[d]	3.27[d]	2.03[d]
	Inoculated	58.20[a]	64.86[a]	69.71[a]	81.53[a]	30.20[a]
Vellayani Jyothika	Uninoculated	3.73[d]	2.20[c]	23.93[c]	26.17[c]	14.39[c]
	Inoculated	9.35[b]	4.32[b]	30.52[b]	32.10[b]	15.53[b]
CD (0.05)		0.654	0.526	1.012	1.517	0.637

*Mean of three replications

Table 11. Changes in polyphenol oxidase activity in response to BlCMV inoculation

Varieties	Treatments	*Polyphenol oxidase activity (changes in absorbance min^{-1} g^{-1} fresh weight)				
		Days after inoculation				
		5	10	15	30	60
CO6	Uninoculated	0.91[c]	0.21[c]	0.21[c]	0.12[d]	0.31[b]
	Inoculated	1.61[a]	0.21[c]	0.21[c]	0.50[a]	4.27[a]
Vellayani Jyothika	Uninoculated	0.41[d]	0.35[b]	0.30[b]	0.25[c]	0.21[b]
	Inoculated	1.22[b]	0.40[a]	0.40[a]	0.31[b]	0.21[b]
CD (0.05)		0.146	0.027	0.029	0.030	0.148

*Mean of three replications

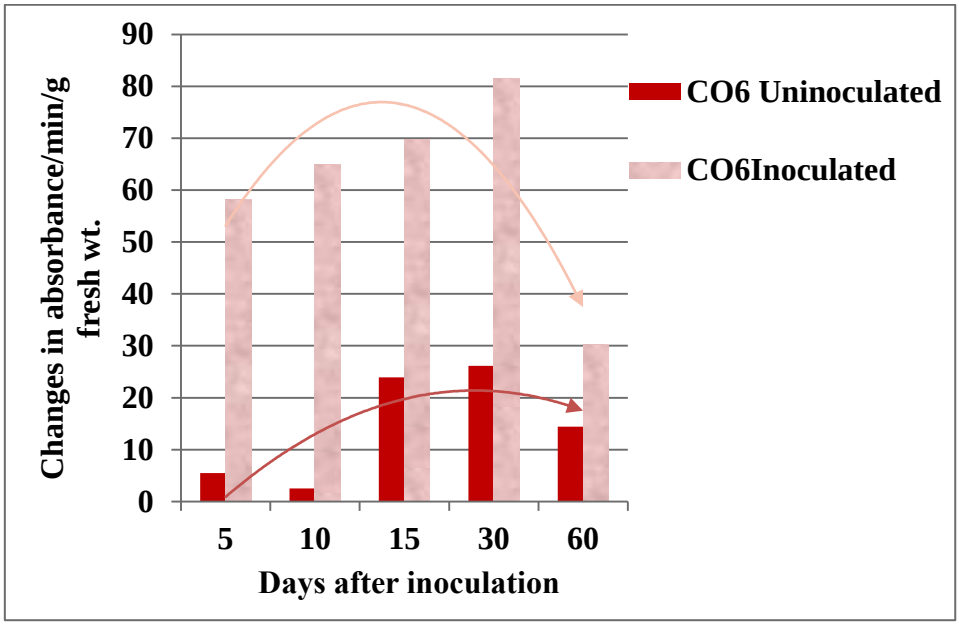

Fig 7a. C06

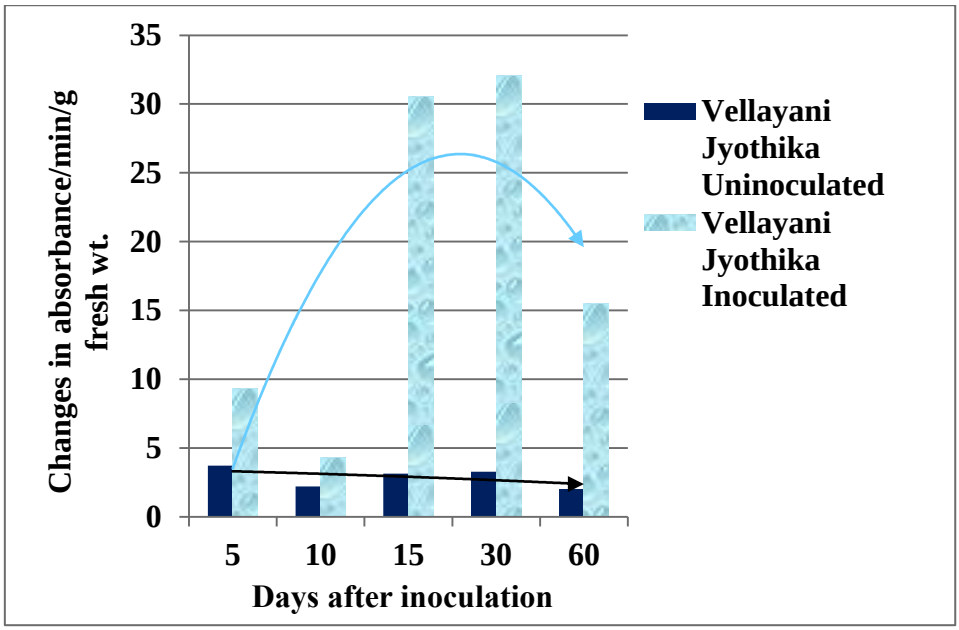

Fig 7b. Vellayani Jyothika

Changes in peroxidase activity in response to BlCMV inoculation

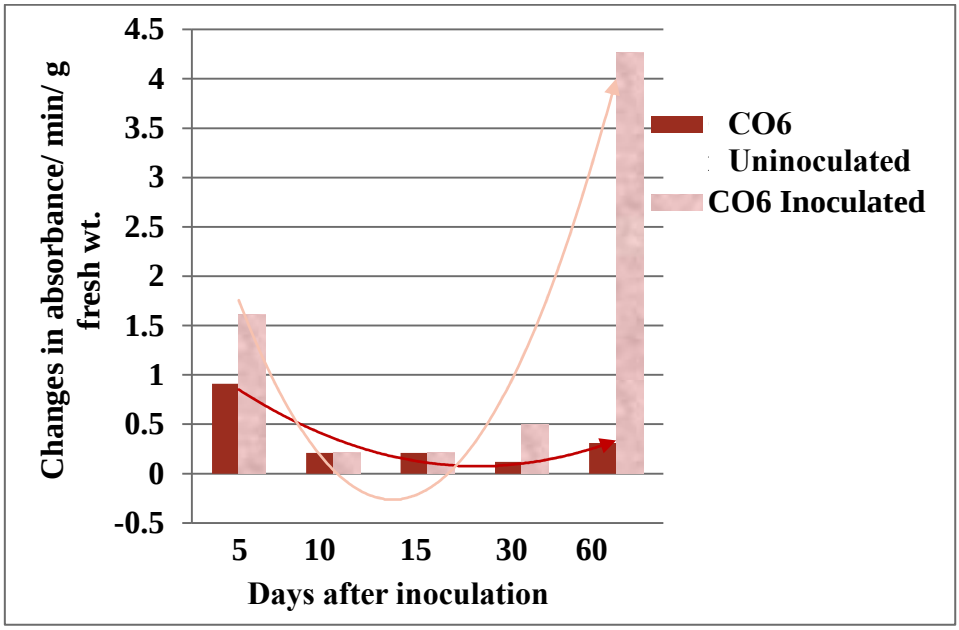

Fig 8a. CO6

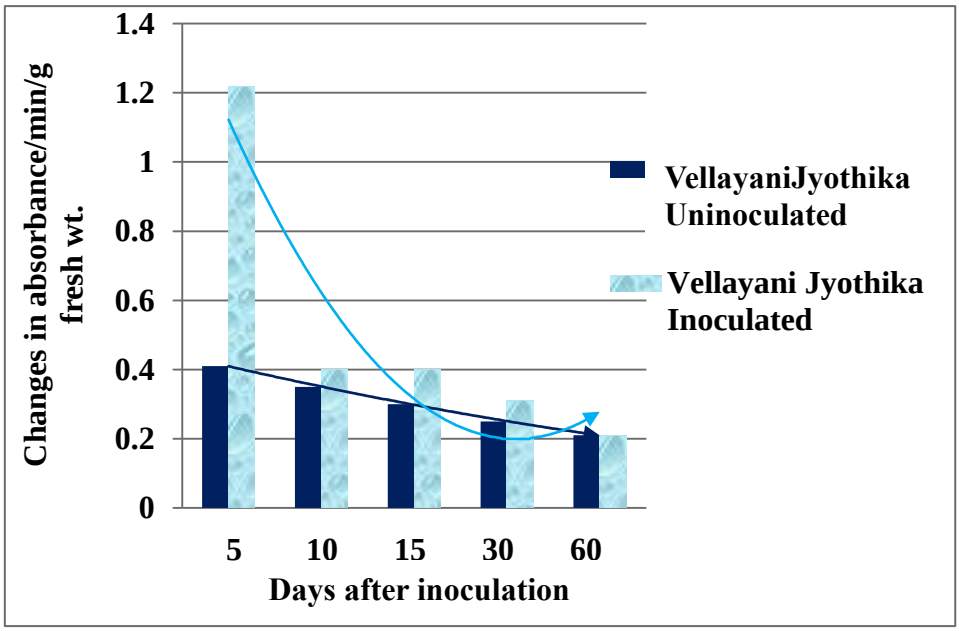

Fig 8b. Vellayani Jyothika

Changes polyphenol oxidase activity in response to BlCMV inoculation

4.1.3.5.3 Estimation of Phenylalanine ammonialyase (PAL)

Phenylalanine ammonialyase activity was estimated as per the procedure developed by Dickerson *et al.* (1984) and results are presented in Table 12 and Fig 9.

In resistant and susceptible genotypes, PAL activity reached the maximum at 5 DAI *i.e.* 80.32 µg g^{-1} min^{-1} and 69.40 µg g^{-1} min^{-1} respectively and there after maintained a lower level over the days of inoculation. The study revealed a significant increase in the PAL activity in the inoculated resistant genotypes compared to the uninoculated and susceptible genotype, throughout the period of observation.

4.1.3.6. Electrophoretic Analysis of Proteins using SDS-PAGE

Protein profiles studies of uninoculated and inoculated cowpea plants of CO6 were analysed and found 9 new proteins were expressed in uninoculated healthy plants (Plate 4a). In inoculated plants, most of proteins were also expressed similar to that of healthy. Plants inoculated at 5, 10 and 15 days after induced 3 (96, 32 and 27 kDa) novel proteins. Samples collected from 30 and 60 DAI, expressed 8 (98, 30, 19, 15, 14, 13, 11 and 8 kDa) additional novel proteins when compared to other treatments.

Protein profiles studies of uninoculated and inoculated cowpea plants of Vellayani Jyothika were analysed and found 9 new proteins in uninoculated healthy plants (Plate 4b). In inoculated plants, most of proteins were also expressed similar to that of healthy. Plants inoculated at 5, 10 and 15 days after induced 3 (97, 43 and 32 kDa) novel proteins. Samples collected from 30 DAI, expressed 7 (22, 20, 18, 14, 11, 10 and 8 kDa) additional novel proteins when compared to other treatments.

Table 12. Changes in phenylalanine ammonialyase activity in response to BlCMV inoculation

Varieties	Treatments	*Phenylalanine ammonialyase activity (changes in absorbance µg g^{-1} min^{-1} fresh weight)				
		Days after inoculation				
		5	10	15	30	60
CO6	Uninoculated	39.95[d]	57.06[b]	44.09[b]	47.22[c]	50.26[d]
	Inoculated	80.32[a]	77.35[a]	55.01[a]	59.65[a]	62.24[a]
Vellayani Jyothika	Uninoculated	64.57[c]	29.40[c]	41.23[c]	46.17[d]	56.10[c]
	Inoculated	69.40[b]	29.40[c]	45.00[b]	50.03[b]	57.10[b]
CD (0.05)		1.314	1.391	1.212	1.172	1.089

*Mean of three replications

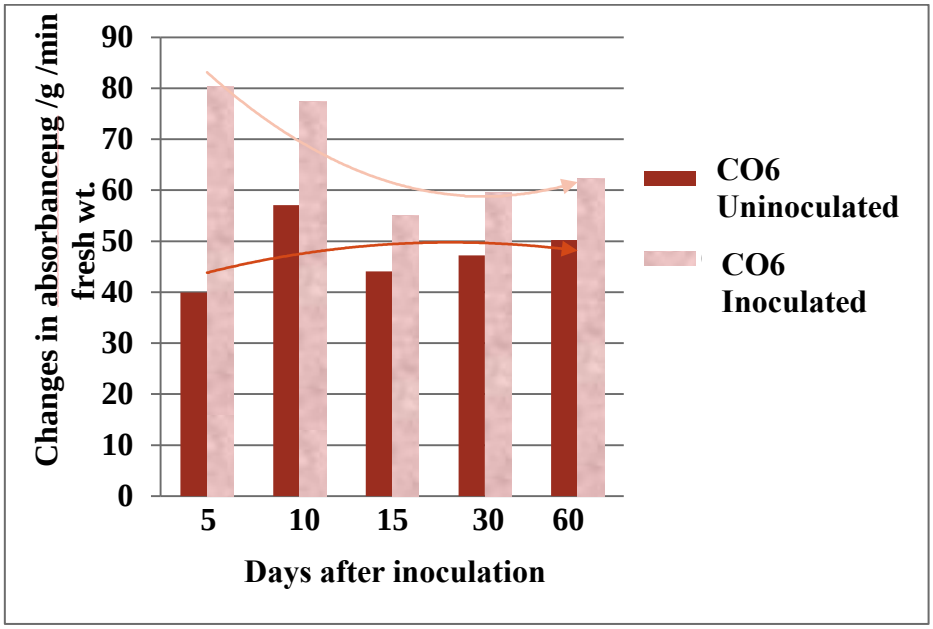

Fig 9a. CO6

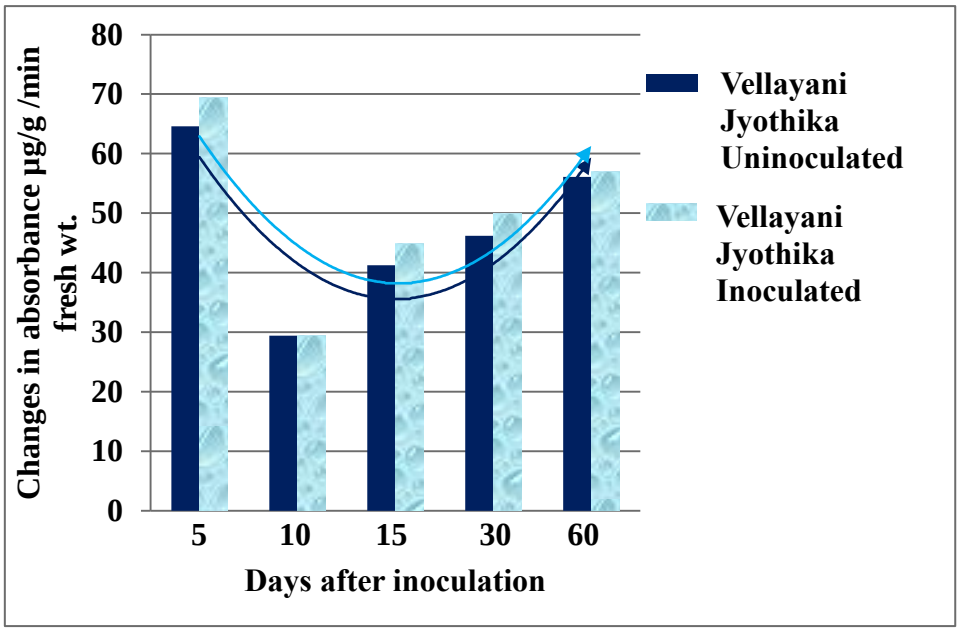

Fig 9b. Vellayani Jyothika

Changes in phenylalanine ammonialyase activity in response to BlCMV inoculation

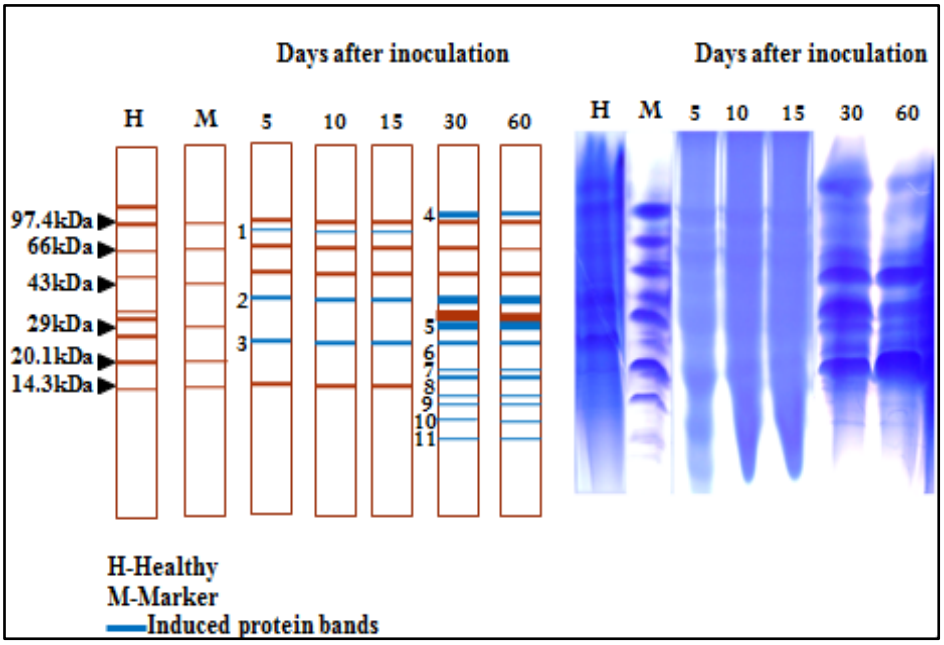

Plate 4a. CO6

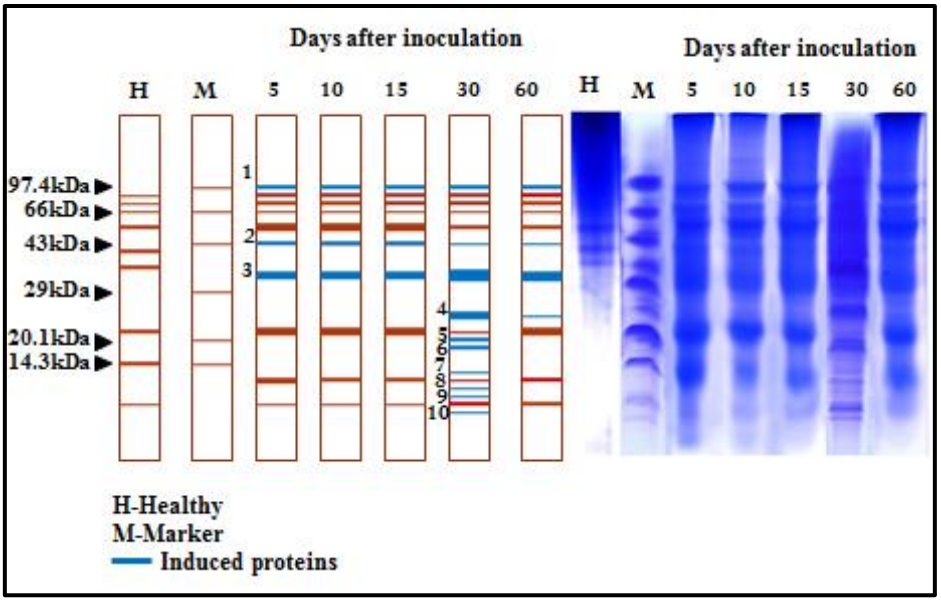

Plate 4b. Vellayani Jyothika

Protein profile in response to BlCMV inoculation

4.1.3.7 Electrophoretic Analysis of Isozyme

4.1.3.7.1 Peroxidase Isozyme Analysis

In the uninoculated plants of resistant genotype, 2 isozymes were detected (a and b) with Rm values of 0.04 and 0.09 respectively (Plate 5a and Table 13). In addition to the two isozymes, CO6 exhibited 4 more isozymes (c, d, e and f) at various stages of inoculation. Isozyme 'c' (Rm value of 0.16) was detected at 5 and 10 DAI. Isozyme'd' (Rm value of 0.20) was detected at 15 DAI. The remaining isozymes (e and f) were identified at 60 DAI with Rm values of 0.13 and 0.27 respectively. It was also noted that, intensity of the isozymes (a and b) enhanced over the days of inoculation, compared to the control.

Uninoculated plants of Vellayani Jyothika recorded 2 isozymes (a and b) with Rm values of 0.04 and 0.09 respectively (Plate 5b and Table 14). Vellayani Jyothika on inoculation, produced one additional isozyme 'c' (Rm value of 0.16) at 15 and 60 DAI. Compared to the healthy plants, there was no change in the intensity of 'a' isozyme, whereas 'b' isozyme enhanced over the days of inoculation.

4.1.4 BIOLOGICAL DETECTION

4.1.4.1 Indicator Hosts for Diagnosis

Attempt was made to inoculate BlCMV to *Chenopodium amaranticolor* and *Chenopodium quinoa.* On inoculation, the development of numerous chlorotic local lesions were observed in *C. amaranticolor,* 5 to 6 DAI (Plate 6). Local lesions later turned necrotic. Mechanical inoculation on *C. quinoa* did not give any symptoms of local lesions. Thus *C. amaranticolor* was identified as the indicator plant for the biological detection of BlCMV.

Table 13. Isoperoxidase activity of CO6 in response to BlCMV inoculation

Treatments	Isoperoxidase activity (*Rm value)					
	Iso PO1 (a)	Iso PO2 (b)	Iso PO3 (c)	Iso PO4 (d)	Iso PO5 (e)	Iso PO6 (f)
Uninoculated control	0.04	0.09	-	-	-	
5 DAI	0.04	0.09	0.16	-	-	-
10 DAI	0.04	0.09	0.16	-	-	-
15 DAI	0.04	0.09	-	0.20	-	-
30 DAI	0.04	0.09	-	-	-	-
60 DAI	0.04	0.09	-	-	0.13	0.27

*Relative mobility

Table 14. Isoperoxidase activity of Vellayani Jyothika in response to BlCMV inoculation

Treatments	Isoperoxidase activity (*Rm value)		
	Iso PO1 (a)	Iso PO2 (b)	Iso PO3 (c)
Uninoculated control	0.04	0.09	-
5 DAI	0.04	-	-
10 DAI	0.04	0.09	-
15 DAI	0.04	0.09	0.16
30 DAI	0.04	0.09	-
60 DAI	0.04	0.09	0.16

*Relative mobility

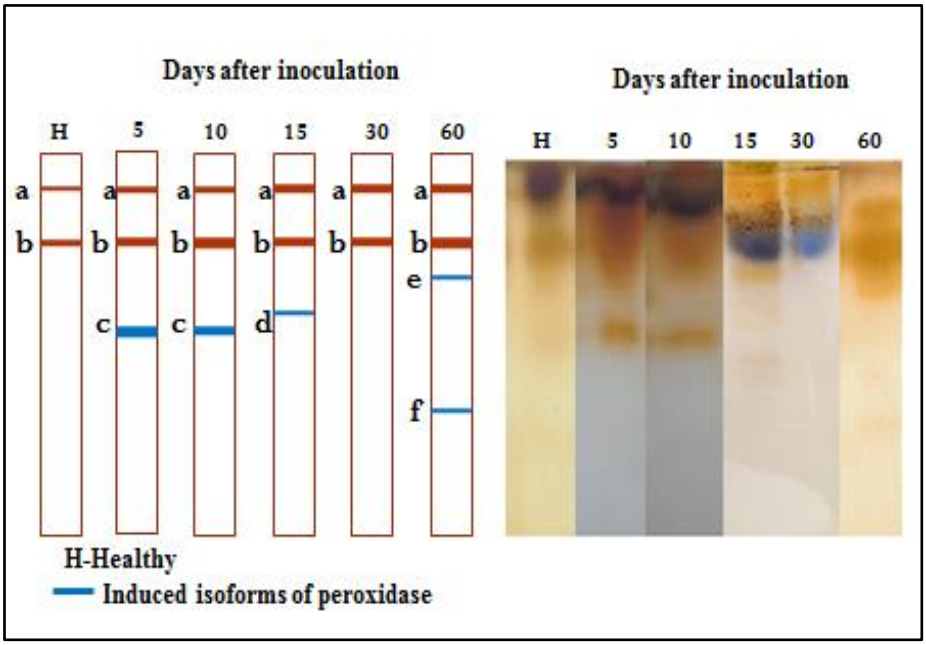

Plate 5a. CO6

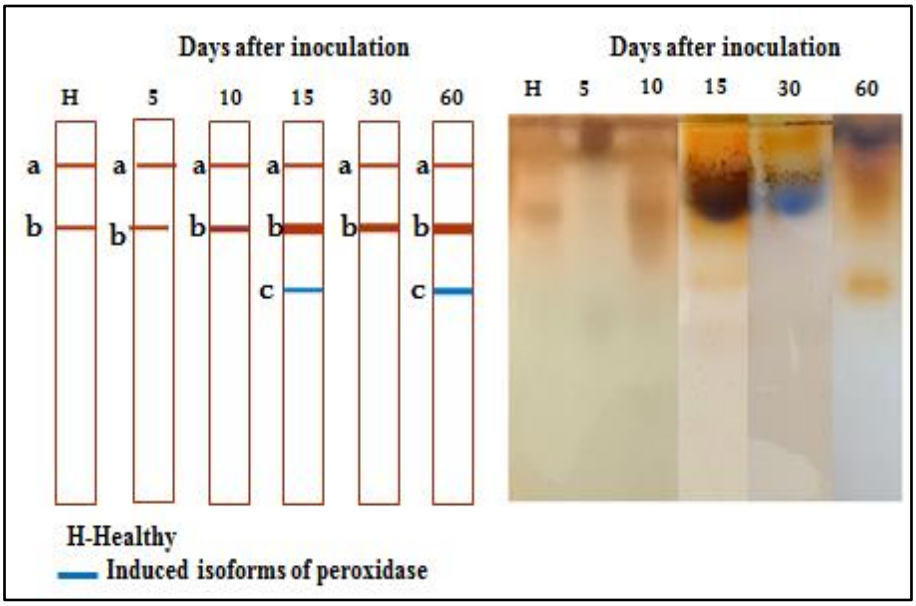

Plate 5b. Vellayani Jyothika

Isoperoxidase profile in response to BlCMV inoculation

4.1.5 IMMUNOLOGICAL STUDIES

4.1.5.1. Enzyme linked immunosorbent assay (ELISA)

4.1.5.1.1. Direct antigen coating-Enzyme linked immunosorbent assay (DAC-ELISA)

DAC-ELISA was used to detect the virus causing mosaic disease in cowpea in Kerala. The absorbance was measured at 405 nm in an ELISA reader (BIO-RAD Microplate Reader 680). Crude sap from infected cowpea leaf tissue was used as antigen source. Polyclonal and monoclonal antibodies specific to BlCMV and *Cowpea aphid-borne mosaic virus* (CABMV) were used for the study. Results are summarised in Tables 15, 16 and 17.

The polyclonal (H2) and two monoclonal antibodies (15E6 and 16G5) against BlCMV gave a positive reaction in samples collected from symptomatic plants (Plate 7). The positive sample (H2) recorded 0.188 and 0.075 for mosaic and vein banding respectively. Monoclonal antibodies (15E6 and 16G5) gave positive results in infected cowpea plants with an absorbance of 0.069 and 0.018 respectively. The leaf extracts of virus infected cowpea did not reacted with the monoclonal and polyclonal antibodies against CABMV.

4.1.5.2 Dot immunobinding assay (DIBA)

DIBA was conducted to detect the presence of the virus causing mosaic disease in cowpea, by using the polyclonal antiserum against BlCMV and CABMV *i.e.* H2 and H4 respectively. It was observed that, antiserum against CABMV gave negative reaction (Plate 8a) whereas BlCMV gave positive reaction (Plate 8b). Hence the inoculum collected from the infected plant samples showed the presence of only the strain of BlCMV and not CABMV. The infected leaf samples showed definite purple coloured spots on nitrocellulose membrane indicating positive reaction, which was absent in healthy and buffer check which was colourless.

Table 15. Reaction of polyclonal antibodies against BlCMV/CABMV in infected cowpea plants

Polyclonal antibodies	Symptoms tested	*Absorbance at 405 nm		Reaction
		Healthy	Infected	
H2 (BlCMV)	Mosaic	0.014	0.188	+
	Vein banding	0.014	0.075	+
H4 (CABMV)	Mosaic	0.014	0.017	-
	Vein banding	0.033	0.062	-

*Mean of three replications

Table 16. Reaction of monoclonal antibodies against BlCMV in infected cowpea plants

Monoclonal antibodies	*Absorbance at 405 nm		Reaction
	Healthy	Infected	
15E6	0.029	0.069	+
16G5	0.007	0.018	+

*Mean of three replications

Table 17. Reaction of monoclonal antibodies against CABMV in infected cowpea plants

Monoclonal antibodies	*Absorbance at 405 nm		Reaction
	Healthy	Infected	
7D9	0.047	0.075	-
IF5	0.027	0.046	-
7A10	0.003	0.004	-
12S9	0.003	0.005	-
5D17	0.031	0.056	-

*Mean of three replications

+ Presence of virus
- Absence of virus

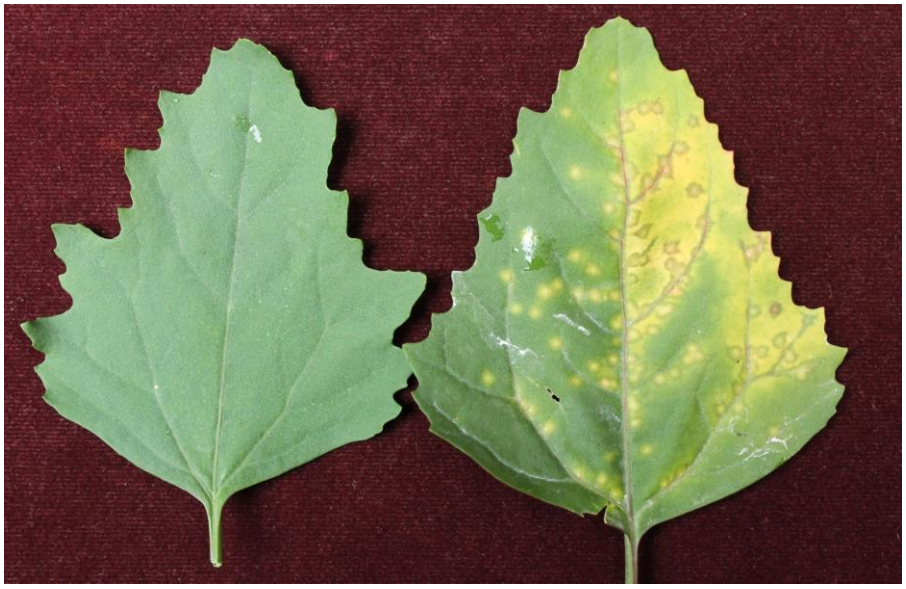

Plate 6. *Chenopodium amaranticolor* on inoculation with BlCMV

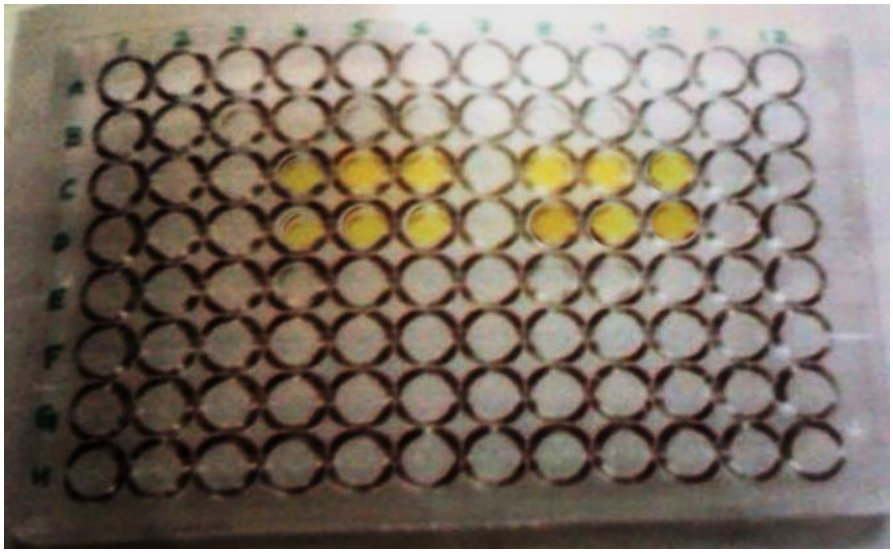

Plate 7. Reaction of BlCMV in DAC-ELISA

Positive reaction to H2: C4, C5 and C6; C8, C9 and C10

Positive reaction to 15E6: D4, D5 and D6

Positive reaction to16G5: D8, D9 and D10

D **B** **H**

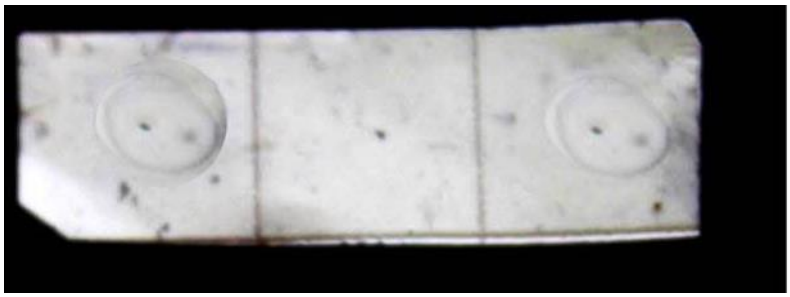

Plate 8a. Negative reaction against CABMV: H4

Plate 8b. Positive reaction against BlCMV: H2

D: Diseased **B: Buffer** **H: Healthy**

Reaction of polyclonal antibodies against BlCMV/CABMV in DIBA

4.1.6 MOLECULAR DETECTION AND CHARACTERISATION

4.1.6.1 Reverse transcription-Polymerase chain reaction (RT-PCR)

Molecular diagnosis using RT-PCR, followed by gel electrophoresis was performed for the specific detection of the viruses causing mosaic disease in cowpea. Primers specifically designed based on the nucleotide sequence of coat protein region of the BlCMV and CABMV were tested. There was no amplification with CABMV specific primer. An amplicon of size 110 bp (Plate 9c), was obtained from the cDNA (Plate 9b) reverse transcribed from viral RNA isolated (Plate 9a), from the infected cowpea leaf sample using the BlCMV specific primer. RT-PCR results revealed that, the mosaic disease of cowpea in Kerala was caused by the *Blackeye cowpea mosaic virus* (BlCMV).

4.1.6.2 Characterisation

The PCR product obtained was sequenced and sequence was analysed using BLAST software (Fig 10 and Table 18). Comparative nucleotide sequence alignment of the viruses with the available data bases from National Centre for Biotechnology Information (NCBI) revealed a 97 per cent homology with *Bean common mosaic virus* (BCMV) strain *Blackeye* from Karnataka. Isolate also shared 93 and 92 per cent homology with two related *Potyviruses viz.* CABMV and *Peanut stripe virus* (PstV) respectively. Comparative amino acid sequence alignment of the isolate revealed a 95 per cent homology with coat protein of BCMV (Fig 11).

4.2 *Papaya ring spot virus* (PRSV)

4.2.1 SYMPTOMATOLOGY

PRSV inoculated plants, of both the genotypes showed a wide range of symptoms. Susceptible genotypes from *Carica papaya* L. initiated disease symptoms as water soaked oily vein clearing symptoms (Plate 10a), on the younger leaves, 14 DAI. Most prominent and characteristic diagnostic symptom noticed was the development of shoe string symptoms (Plate 10b) on the leaves,

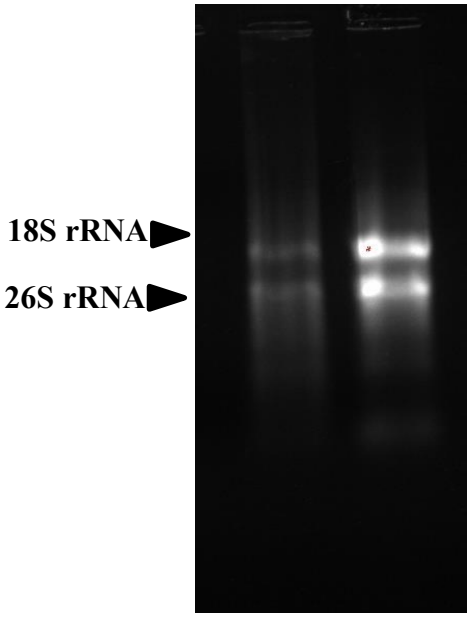

Plate 9a. Total RNA

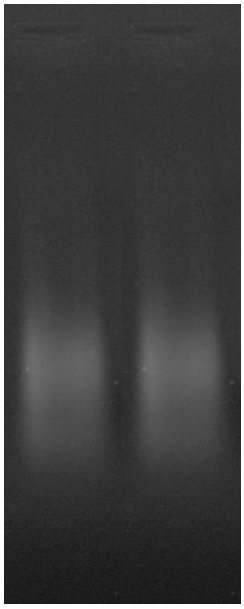

Plate 9b. cDNA

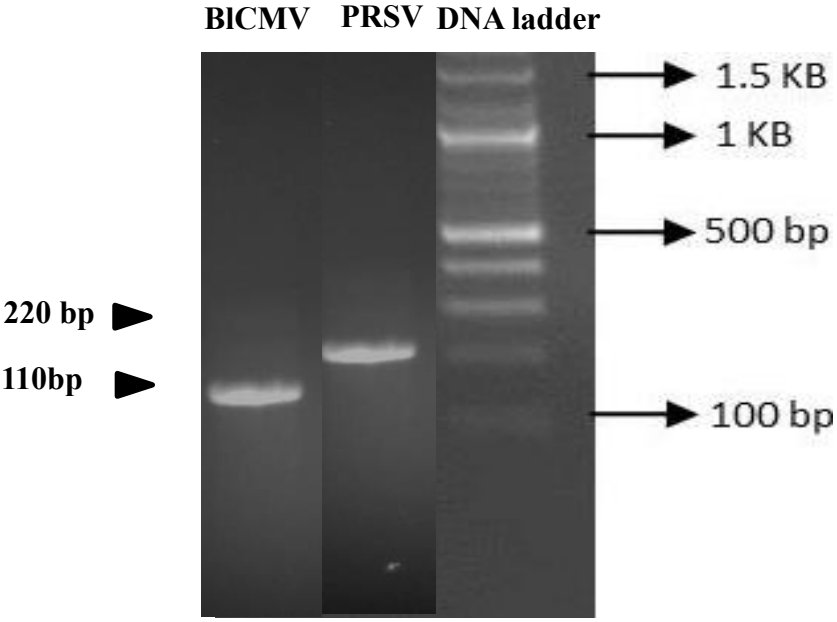

Plate 9c. RT-PCR Amplification

Molecular diagnosis of BlCMV

Table 18. Comparative nucleotide sequence alignment of BlCMV isolate with reported isolates of *Potyviruses*

Accession No.	Description	Nucleotide identity (%)
DQ167404.1	*Bean common mosaic virus* strain Blackeye coat protein-like gene, partial sequence	97
KC871565.1	*Bean common mosaic virus* isolate Coochbehar polyprotein gene, partial cds	97
AY575773.1	*Blackeye cowpea mosaic virus*, complete genome	97
HQ864647.1	*Bean common mosaic virus* isolate Panthnagar polyprotein-like mRNA, partial sequence	96
HQ864645.1	*Bean common mosaic virus* isolate Hempur polyprotein-like Mrna, partial sequence	96
HQ864644.1	*Bean common mosaic virus* isolate Mysore polyprotein-like Mrna, partial sequence	96
U72204.1/CAU72204	*Cowpea aphid-borne mosaic virus* Nib protein/coat protein gene, complete cds	93
Q753313.1	*Bean common mosaic virus* isolate Sikkim coat protein (CP) gene, partial cds	94
DQ167406.1	*Bean common mosaic virus* isolate Mysore coat protein-like gene, partial cds	94
AJ132158.1	*Peanut stripe virus* polyprotein gene 3' terminus, isolate Indonesia	92

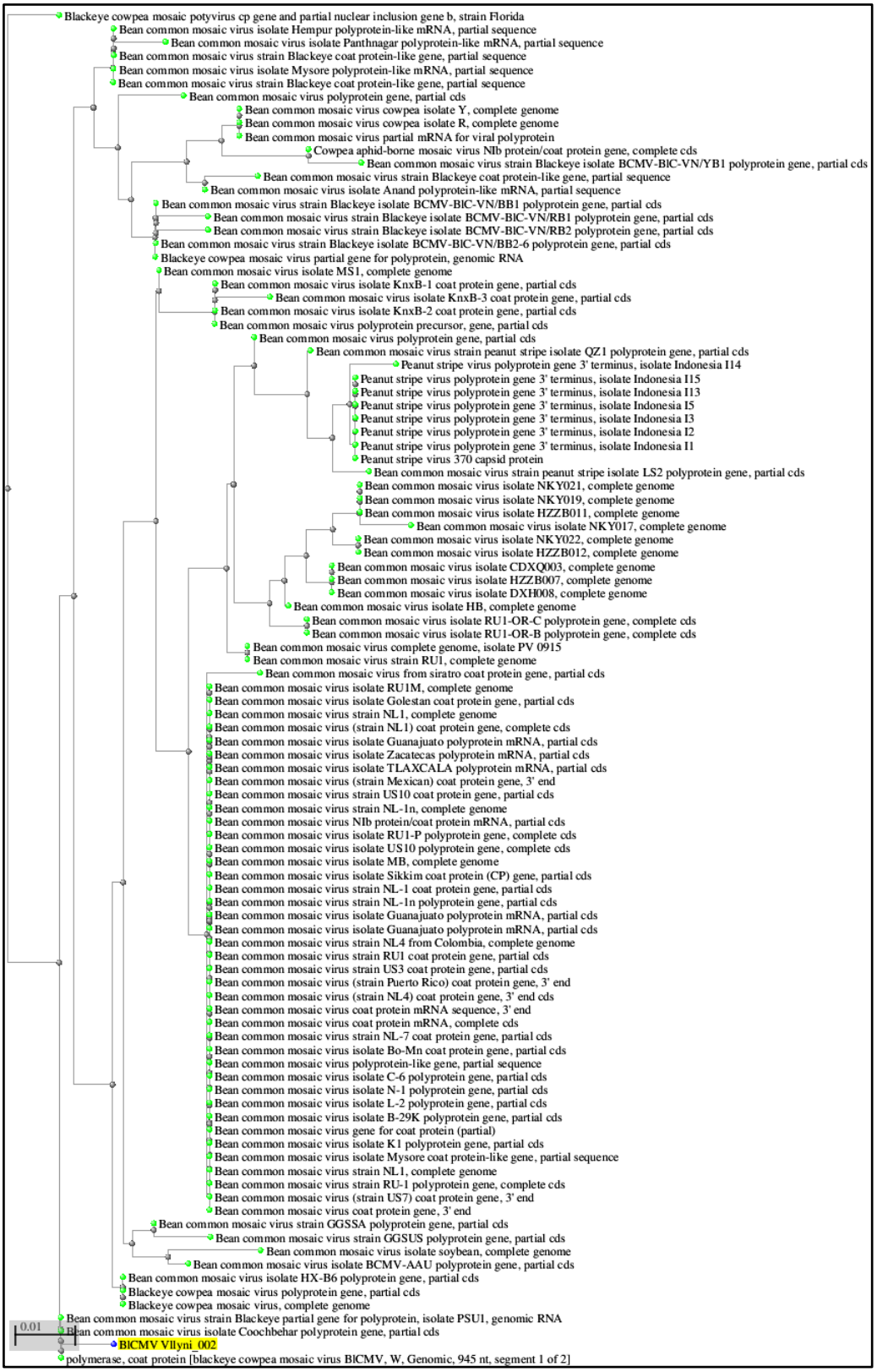

Fig 10. Dendrogram showing the relationship of Vellayani isolate of BlCMV with related *Potyviruses*

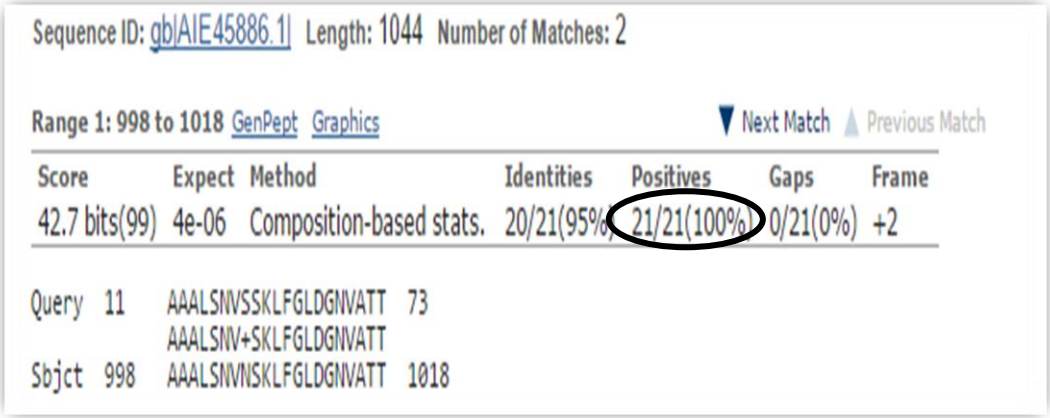

Fig 11. Comparative amino acid sequence alignment of Vellayani isolate of BlCMV with its coat protein sequence

were in the leaf lamina was highly reduced and narrowed down, resembling a shoe string. The mosaic patterns (Plate 10c) were also observed, sometimes localised or covering the complete leaf area. Leaves also showed puckering (Plate 10d), with dark green raised areas demarcated by veins seen either localised or affecting the complete leaf. A few leaves also showed vein banding symptoms. Complete rings or broken rings and chlorotic spots were observed on leaves (Plate 10e) and fruits (Plate 10f) typical of Papaya ring spot disease. Infected leaf became completely distorted and disfigured, with marked size reduction.

All the mechanically inoculated plants of resistant genotype, Pusa Nanha showed symptom development, 20 DAI. PRSV symptoms on the resistant genotype started as mild mosaic and leaf chlorosis. These symptoms were developed on few leaves of the mechanically inoculated plants. There was no increase in the disease severity till the end of the experiment (60 DAI).

Production of flowers and fruits in susceptible varieties were also severely reduced. Fruits of the infected trees were disfigured with development of bumps on them. There was severe reduction in height of the tree and size of the canopy. Severe disease incidence even resulted in stunted growth (Plate 10g) and complete death of the tree (Plate 10h).

4.2.2 TRANSMISSION STUDIES

Transmission studies were conducted using a susceptible local variety of papaya.

4.2.2.1 Mechanical Transmission

Sap transmission of PRSV to healthy papaya plants was done at six leaf stage using 0.1 *M* Sodium phosphate buffer (pH 7.0). The initial symptoms of oily vein clearing was noticed on the newly emerging leaves, 14 DAI with 100 per cent transmission (Table 19).

Plate 10a. Vein clearing

Plate 10b. Shoestring

Plate 10c. Mosaic

Plate 10d. Puckering

Symptoms of PRSV infection in papaya

Plate 10e. Ring spots on leaf

Plate 10f. Ring spots on fruit

Plate 10g. Stunted growth

Plate 10h. Death of tree

Symptoms of PRSV infection in papaya

4.2.2.2 Seed Transmission

Transmission of PRSV through seeds from papaya plants was studied. No seed transmission was recorded for PRSV up to sixty days after sowing (DAS) (Table 20).

4.2.2.3 Insect Transmission

Attempt was made to transmit PRSV through insects associated with papaya namely aphids and papaya mealy bug. Aphid species *viz*. *Aphis craccivora* (Plate 11a) and *Aphis gossypii* (Plate 11b) successfully transmitted the virus, with 30 per cent and 40 per cent transmission, respectively. Papaya mealy bug (*Paracoccus marginatus* Williams and Garnara de Willink) (Plate 11c) proved to be the most efficient vector, with 80 per cent transmission followed by *A. gossypii* (40 per cent) and *A. craccivora* (30 per cent) (Table 21).

4.2.3 HOST PATHOGEN INTERACTION STUDIES

Study was conducted to document the effects of PRSV on the total chlorophyll and carbohydrate contents of the resistant genotype, Pusa Nanha (Plate 12b) and a susceptible local variety of papaya (Plate 12a), at different stages of inoculation. Quantitative estimations of phenol, protein content, peroxidase, polyphenol oxidase and phenylalanine ammonialyase activities, at different stages of inoculation, were also analysed to find their possible role in inducing defense responses against PRSV.

The data collected from the experiments was analysed separately for each parameter and subjected to simple CRD for each day of inoculation.

4.2.3.1 Estimation of Carbohydrate

The observation of experiment are summarised in Table 22 and Fig 12.

Pusa Nanha on inoculation, recorded the highest carbohydrate content at 5 DAI (22.97 mg g^{-1}), which declined at 60 DAI (8.02 mg g^{-1}). It was observed that susceptible local variety on inoculation, recorded the maximum carbohydrate

Table 19. Mechanical transmission studies of PRSV

Sl. No.	Test plant	No. of plants inoculated	No. of plants infected	Per cent transmission
1	Papaya	100	100	100

Table 20. Seed transmission studies of PRSV

Sl. No.	Seeds obtained from	No. of seeds sown from infected plants	No. of plants infected	Per cent transmission
1	Healthy papaya	25	0	0
2	Diseased papaya	25	0	0

Table 21. Insect transmission studies of PRSV

Sl. No.	Insect species	No. of insects released per plant	No. of plants inoculated	No. of plants infected	Per cent transmission
1	*Aphis craccivora*	10	10	3	30
2	*Aphis gossypii*	10	10	4	40
3	*Paracoccus marginatus*	10	10	8	80

Plate 11a. ***Aphis gossypii*** **Glover**

Plate 11b. ***Aphis craccivora*** **Koch**

Plate 11c. ***Paracoccus marginatus*** **Williams and Garnara de Willink**

Insect vectors used for transmission study of PRSV

Plate 12a. Pusa Nanha

Plate 12b. Local variety

Varieties of papaya used for the study

content at 10 DAI (17.40 mg g^{-1}) which declined at 60 DAI (9.32 mg g^{-1}). The amount of total carbohydrates was found to decrease significantly in inoculated plants of resistant and susceptible genotypes. However, the carbohydrate content of inoculated resistant genotype was significantly higher than the inoculated susceptible genotype throughout the period of observation.

4.2.3.2 Estimation of Chlorophyll

Chlorophyll a, Chlorophyll b and total chlorophyll contents of inoculated and uninoculated plants were determined and are presented in the Tables 23, 24 and 25 and Figs 13, 14 and 15.

Chlorophyll a content of the inoculated plants of Pusa Nanha recorded the maximum value at 5 DAI (1.62 mg g^{-1}), which declined at 60 DAI (1.14 mg g^{-1}). However, the inoculated plants of susceptible variety showed an increasing trend, over days of inoculation and reached the maximum value at 30 DAI (1.19 mg g^{-1}). There after the content decreased. Chlorophyll b content of both the genotypes showed an increasing over the days of inoculation and reached the maximum at 60 DAI (1.91 mg g^{-1}) for Pusa Nanha and 30 DAI (0.41 mg g^{-1}) for the local variety. Total chlorophyll content of Pusa Nanha increased from 5 DAI (2.17 mg g^{-1}) to 60 DAI (3.05 mg g^{-1}), whereas local variety recorded only a mild increase from 5 DAI (1.01 mg g^{-1}) to 30 DAI (1.60 mg g^{-1}), there after showed a marked reduction. Chlorophyll a, chlorophyll b and total chlorophyll contents of inoculated plants of Pusa Nanha were significantly higher than the inoculated plants of susceptible local variety. Total chlorophyll content of inoculated plants of both genotypes showed a significant decrease compared to the uninoculated plants.

4.2.3.3 Estimation of Phenol

Phenol content of inoculated and uninoculated plants of Pusa Nanha and susceptible genotype are presented in the Table 26 and Fig 16.

Pusa Nanha on inoculation exhibited a significant increase in phenol content over the uninoculated plants with the maximum value recorded at 10 DAI (10.21 mg g^{-1}). However, it was found to differ non-significantly in the leaves of inoculated plants of susceptible genotype, with maximum value recorded at 60 DAI (5.51 mg g^{-1}). Except at 5 DAI, at all the other stages of inoculation, the content of total phenols was significantly higher in leaves of inoculated plants of Pusa Nanha, compared to the uninoculated plants and susceptible genotype.

4.2.3.4 Estimation of Total Soluble Protein

In the resistant genotype, the total protein content showed an increasing trend over days of inoculation, whereas the susceptible local variety exhibited a decreasing trend (Table 27 and Fig 17).

In Pusa Nanha the protein content was found to increase from 5 DAI (1.87 mg g^{-1}) and attained a peak at 60 DAI (7.09 mg g^{-1}). In the case of susceptible variety, protein content declined from 5 DAI (7.40 mg g^{-1}) to 60 DAI (3.83 mg g^{-1}). A significant increase in protein was noticed for the inoculated plants of Pusa Nanha compared to uninoculated plants throughout the period of observation.

4.2.3.5 Estimation of Defense Related Enzymes

4.2.3.5.1 Estimation of Peroxidase (PO)

The changes in enzyme activity on the inoculated and uninoculated plants of both genotypes are presented in the Table 28 and Fig 18.

In Pusa Nanha, PO activity increased from 5 DAI (9.62 min^{-1} g^{-1}) and reached the maximum at 10 DAI (20.42 min^{-1} g^{-1}), which declined there after. Susceptible variety on inoculation recorded the maximum activity at 5 DAI (25.90 min^{-1} g^{-1}) which showed a gradual decline over the days of inoculation. The PO activity was significantly higher in inoculated plants of Pusa Nanha, compared to uninoculated plants and the susceptible genotype except 5 DAI. The inoculated plants of susceptible genotype, recorded an initial significant increase in the enzyme activity which declined at 30 and 60 DAI.

Table 22. Changes in total carbohydrate content in response to PRSV inoculation

Varieties	Treatments	*Total carbohydrate content (mg g^{-1} fresh weight)				
		Days after inoculation				
		5	10	15	30	60
Pusa Nanha	Uninoculated	59.14[a]	47.99[a]	27.20[b]	7.06[b]	9.17[b]
	Inoculated	22.97[c]	19.99[c]	9.90[c]	6.15[c]	8.02[c]
Local variety	Uninoculated	30.02[b]	38.20[b]	29.18[a]	10.93[a]	10.02[a]
	Inoculated	9.17[d]	17.40[d]	9.07[d]	7.03[b]	9.32[b]
CD (0.05)		0.445	0.680	0.228	0.462	0.509

*Mean of three replications

Table 23. Changes in chlorophyll a content in response to PRSV inoculation

Varieties	Treatments	*Chlorophyll a content (mg g^{-1} fresh weight)				
		Days after inoculation				
		5	10	15	30	60
Pusa Nanha	Uninoculated	1.94[a]	1.60[a]	1.65[a]	2.24[a]	1.31[a]
	Inoculated	1.62[b]	1.37[b]	1.23[b]	1.31[a]	1.14[b]
Local variety	Uninoculated	1.02[c]	1.37[b]	1.79[c]	2.18[b]	0.83[c]
	Inoculated	0.74[d]	1.05[c]	1.14[c]	1.19[b]	0.61[d]
CD (0.05)		0.061	0.065	0.108	0.151	0.099

*Mean of three replications

Table 24. Changes in chlorophyll b content in response to PRSV inoculation

Varieties	Treatments	*Chlorophyll b content (mg g^{-1} fresh weight)				
		Days after inoculation				
		5	10	15	30	60
Pusa Nanha	Uninoculated	0.65[a]	0.61[a]	0.55[a]	0.73[a]	2.32[a]
	Inoculated	0.55[b]	0.52[b]	0.48[b]	0.66[b]	1.91[b]
Local variety	Uninoculated	0.34[c]	0.481[c]	0.58[a]	0.68[b]	1.80[c]
	Inoculated	0.27[d]	0.40[d]	0.41[c]	0.41[c]	0.20[d]
CD (0.05)		0.038	0.015	0.047	0.030	0.013

*Mean of three replications

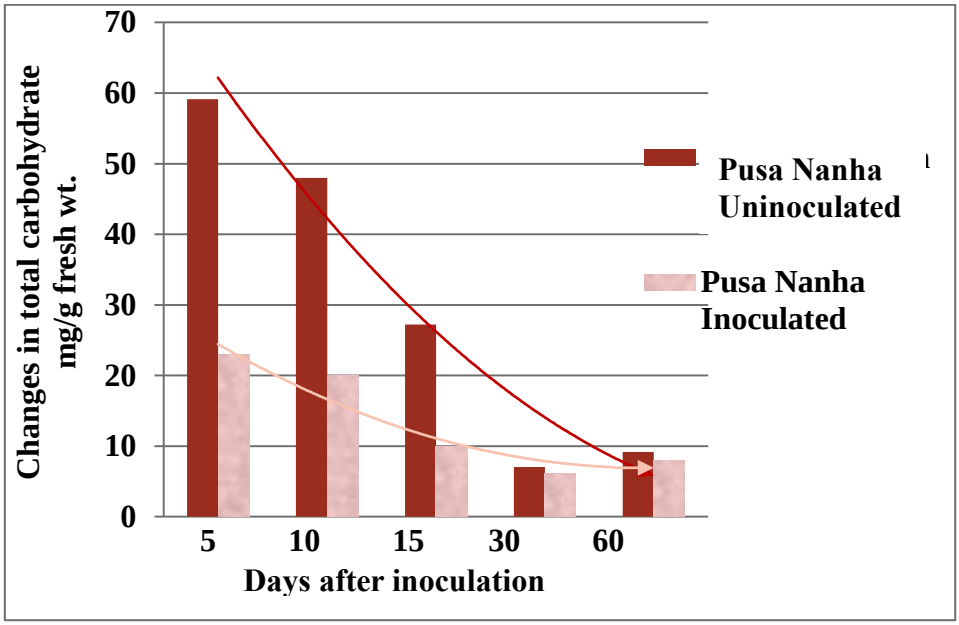

Fig 12a. Pusa Nanha

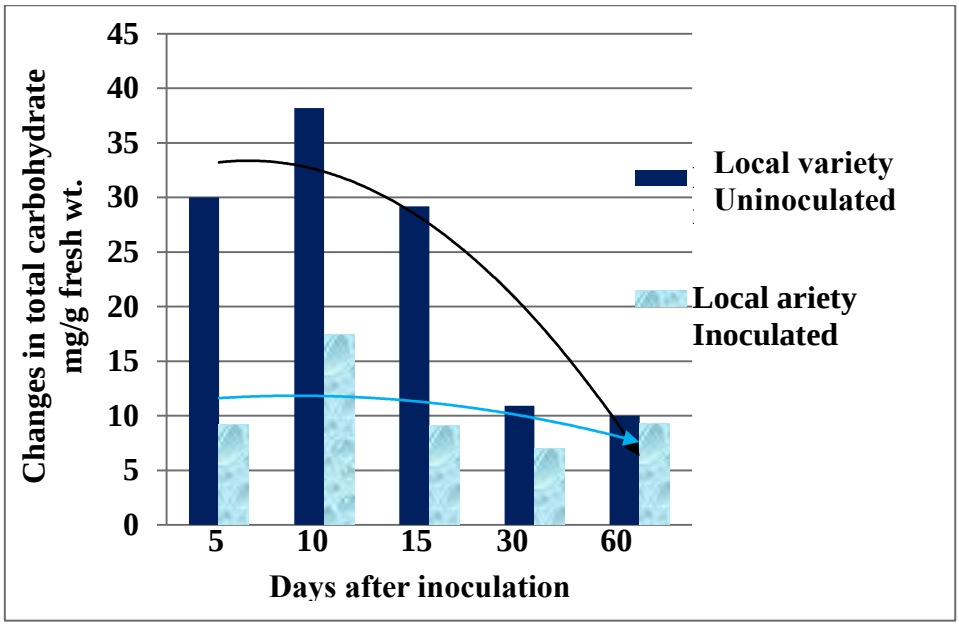

Fig 12b. Local variety

Changes in total carbohydrate content in response to PRSV inoculation

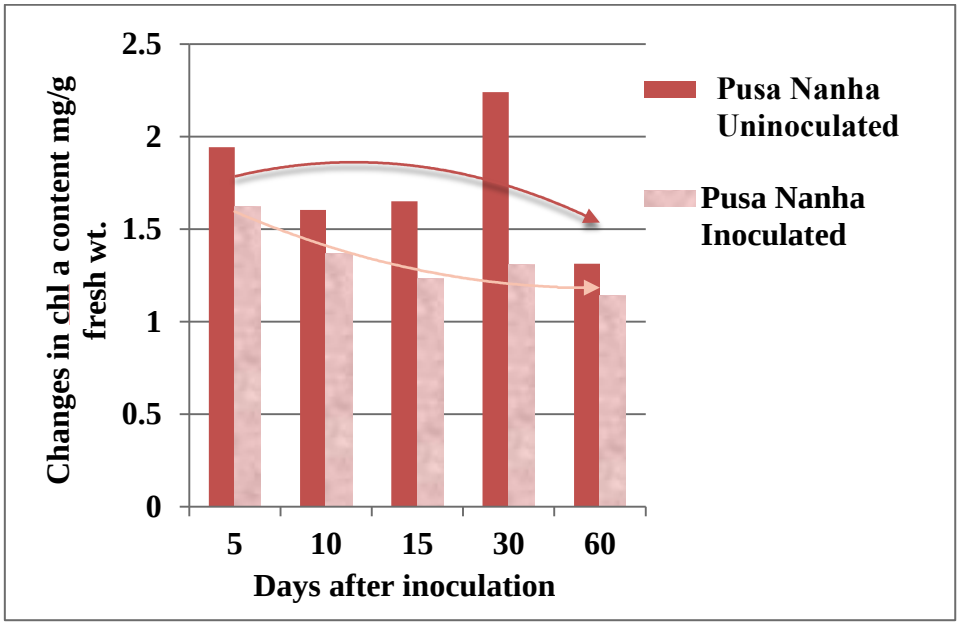

Fig 13a. Pusa Nanha

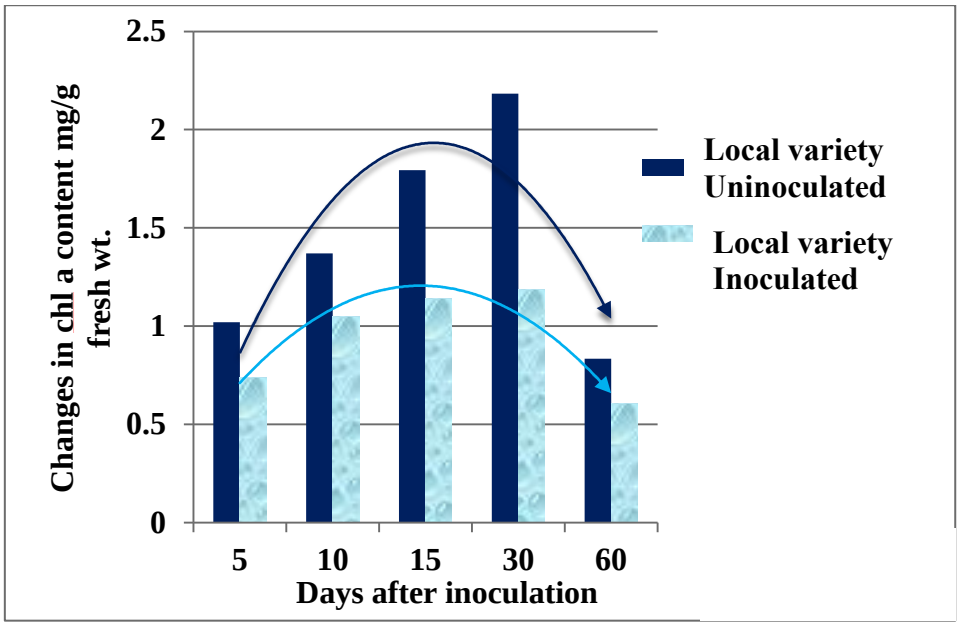

Fig 13b. Local variety

Changes in chlorophyll a content in response to PRSV inoculation

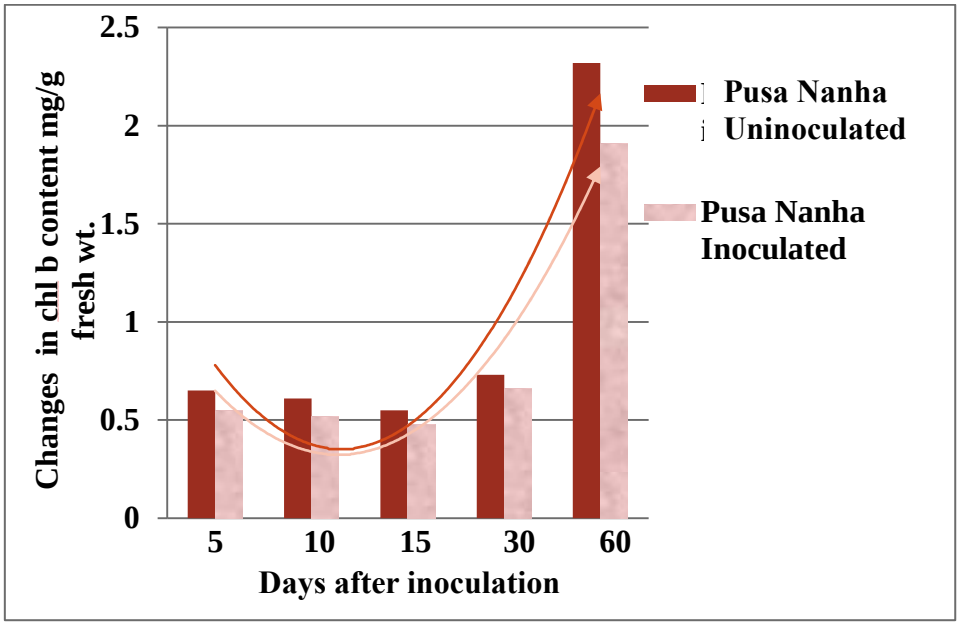

Fig 14a. Pusa Nanha

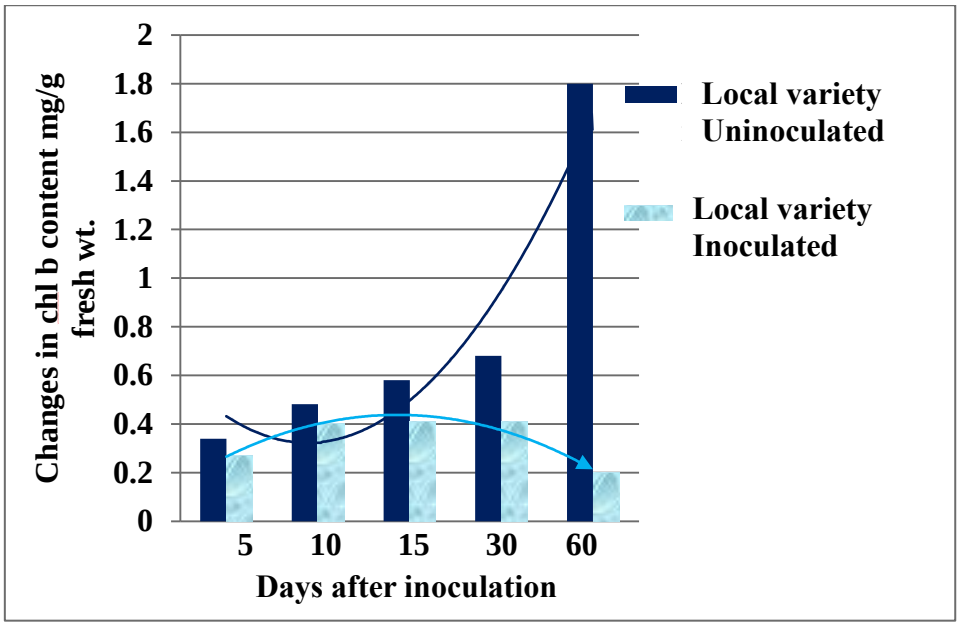

Fig 14b. Local variety

Changes in chlorophyll b content in response to PRSV inoculation

Table 25. Changes in total chlorophyll content in response to PRSV inoculation

Varieties	Treatments	*Total chlorophyll content (mg g^{-1} fresh weight basis)				
		Days after inoculation				
		5	10	15	30	60
Pusa Nanha	Uninoculated	2.59^{a}	2.21^{a}	2.20^{b}	2.97^{a}	3.63^{a}
	Inoculated	2.17^{b}	1.89^{b}	1.71^{c}	1.97^{c}	3.05^{b}
Local variety	Uninoculated	1.36^{c}	1.85^{c}	2.37^{a}	2.86^{b}	2.63^{c}
	Inoculated	1.01^{d}	1.45^{d}	1.55^{d}	1.60^{d}	0.81^{d}
CD (0.05)		0.018	0.026	0.095	0.012	0.018

*Mean of three replications

Table 26. Changes in phenol content in response to PRSV inoculation

Varieties	Treatments	* Phenol content (mg g^{-1} fresh weight basis)				
		Days after inoculation				
		5	10	15	30	60
Pusa Nanha	Uninoculated	2.40^{c}	9.39^{b}	4.61^{b}	2.43^{b}	6.23^{b}
	Inoculated	3.21^{b}	10.21^{a}	6.97^{a}	3.03^{a}	9.22^{a}
Local variety	Uninoculated	5.14^{a}	4.82^{c}	4.46^{b}	1.85^{c}	5.46^{c}
	Inoculated	5.20^{a}	4.92^{c}	4.51^{b}	1.95^{c}	5.51^{c}
CD (0.05)		0.221	0.196	0.265	0.200	0.099

*Mean of three replications

Table 27. Changes in total soluble protein content in response to PRSV inoculation

Varieties	Treatments	*Total soluble protein content (mg g^{-1} fresh weight)				
		Days after inoculation				
		5	10	15	30	60
Pusa Nanha	Uninoculated	1.40^{d}	1.70^{c}	2.15^{c}	3.23^{b}	5.23^{b}
	Inoculated	1.87^{c}	1.89^{b}	2.37^{b}	4.09^{a}	7.09^{a}
Local variety	Uninoculated	7.80^{a}	3.11^{a}	2.73^{a}	2.66^{d}	2.84^{d}
	Inoculated	7.40^{b}	3.15^{a}	2.80^{a}	3.06^{c}	3.83^{c}
CD (0.05)		0.092	0.137	0.202	0.132	0.189

*Mean of three replications

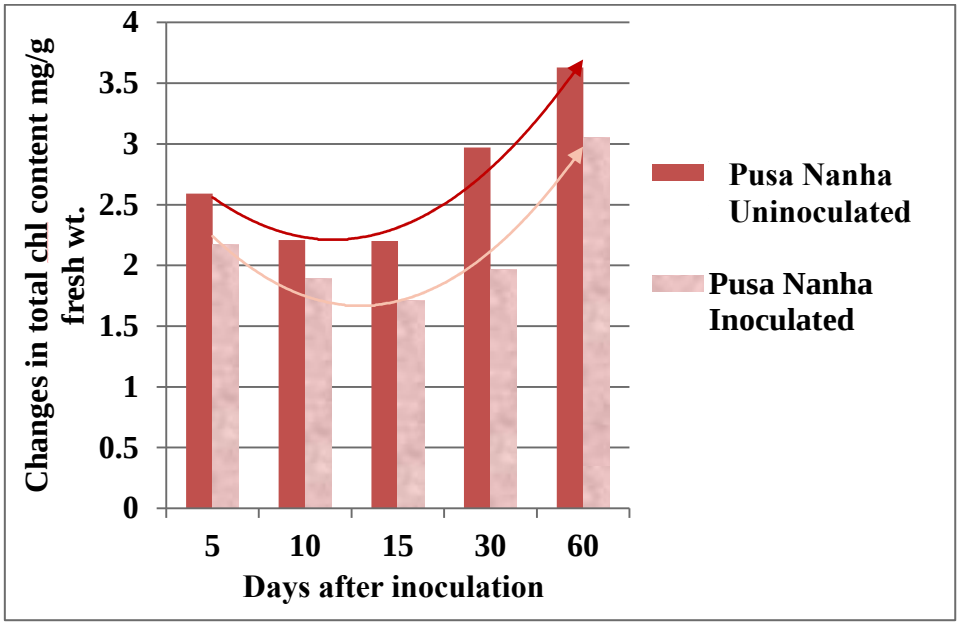

Fig 15a. Pusa Nanha

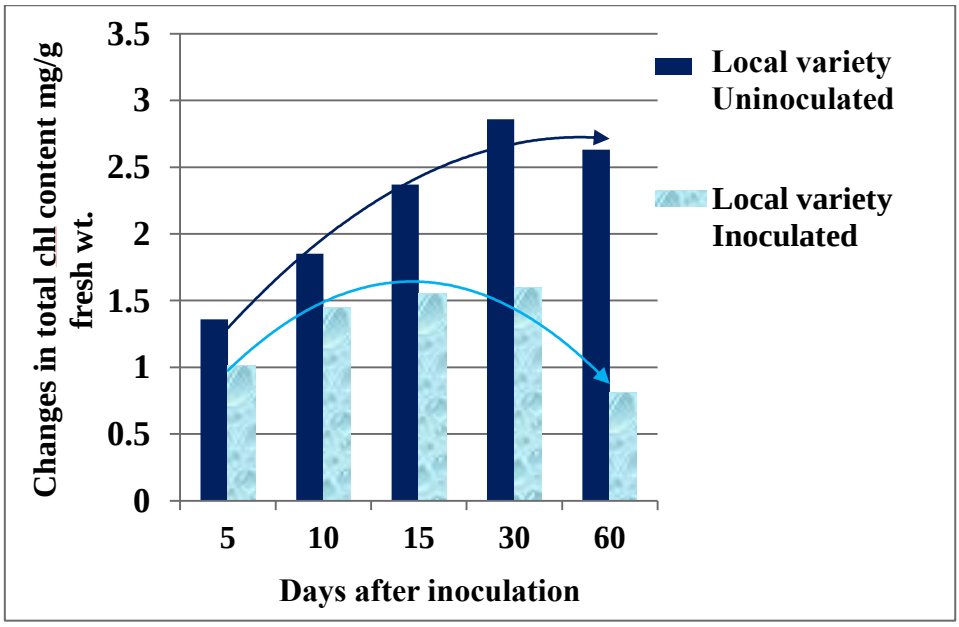

Fig 15b. Local variety

Changes in total chlorophyll content in response to PRSV inoculation

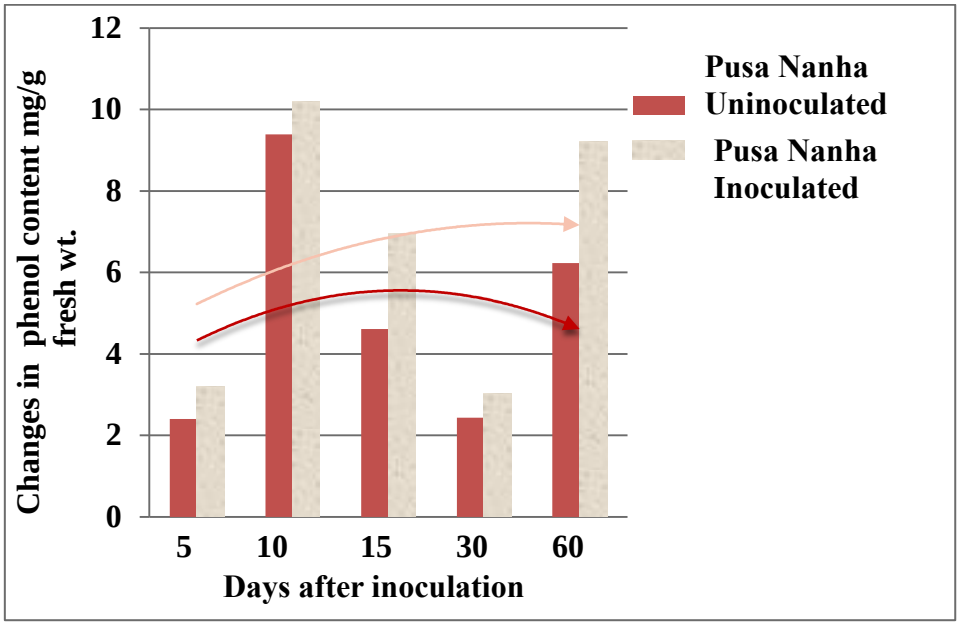

Fig 16a. Pusa Nanha

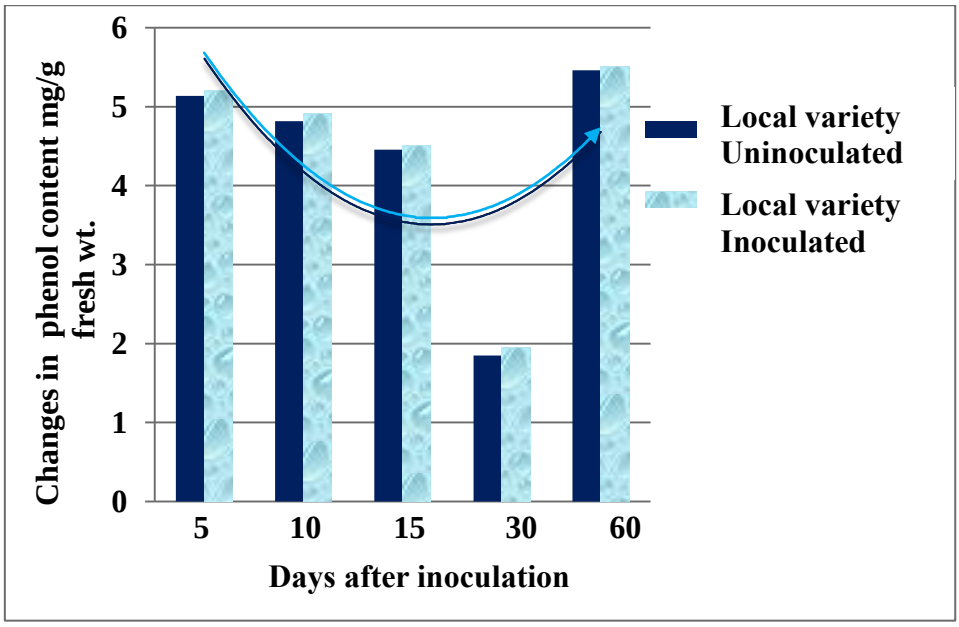

Fig 16b. Local variety

Changes in phenol content in response to PRSV inoculation

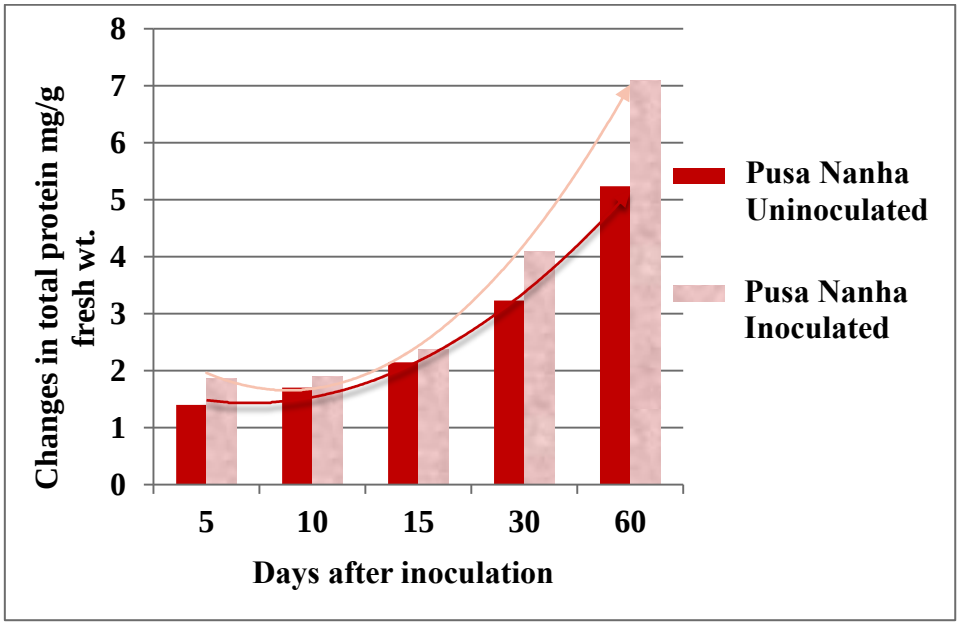

Fig 17a. Pusa Nanha

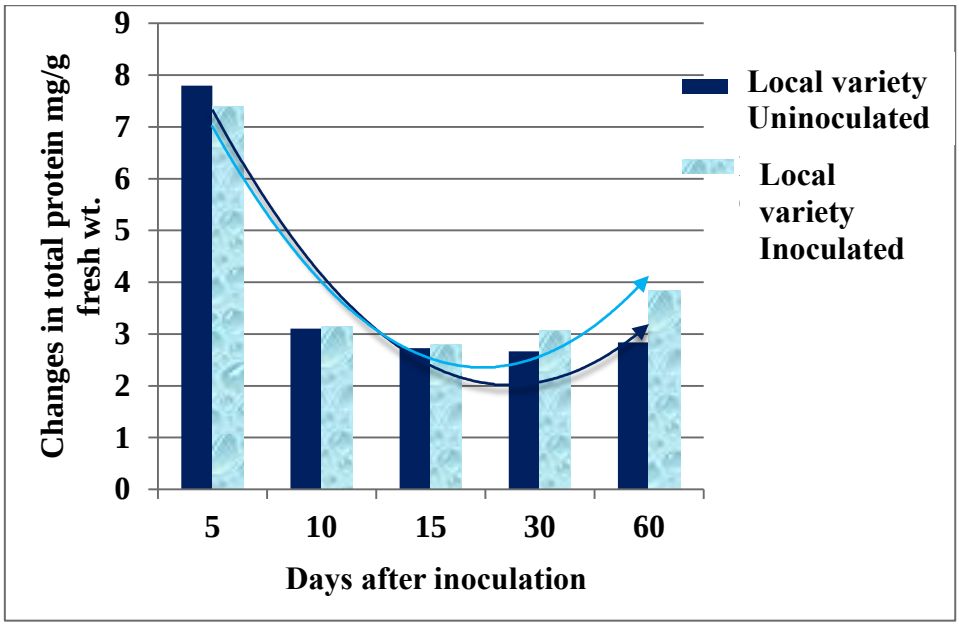

Fig 17b. Local variety

Changes in total soluble protein content in response to PRSV inoculation

4.2.3.5.2 Estimation of Polyphenol oxidase (PPO)

The results of the experiment are presented in Table 29 and Fig 19.

The PPO activity was found to increase significantly in the inoculated plants of Pusa Nanha, except 5 DAI, compared to the susceptible genotype. The maximum activity was observed at 10 DAI (0.50 min^{-1} g^{-1}) which reduced to a constant value at 30 and 60 DAI (0.21 min^{-1} g^{-1}). The enzyme activity showed a non-significant difference in the inoculated plants of susceptible variety, compared to the uninoculated counterparts. Here the maximum activity was noticed at 5 DAI (0.31 min^{-1} g^{-1}) and declined to a constant value at 15, 30 and 60 DAI (0.11 min^{-1} g^{-1}).

4.2.3.5.3 Estimation of Phenylalanine ammonialyase (PAL)

The findings of experiment are summarised in Table 30 and Fig 20.

PAL activity in Pusa Nanha showed an increase from 5 DAI (98.02 µg $g^{-1}min^{-1}$) and reached the maximum at 15 DAI (106.10 µg $g^{-1}min^{-1}$) and declined there after. The inoculated plants of the susceptible local variety recorded the highest activity at 5 DAI (76.64 µg g^{-1} min^{-1}), which maintained a lower level throughout the period of observation. The results revealed that, enzyme activity was significantly higher in the inoculated plants of Pusa Nanha, compared to the uninoculated plants and inoculated susceptible genotype. On the other hand the activity was found to decrease significantly in the inoculated plants of susceptible genotypes, compared to the uninoculated control plants.

4.2.3.6 Electrophoretic Analysis of Proteins using SDS-PAGE

Protein profiles studies of uninoculated and inoculated papaya plants of Pusa Nanha were analysed and found 5 new proteins in uninoculated healthy plants (Plate 13a). In inoculated plants, most of proteins were also expressed similar to that of healthy. Plants inoculated 5, 10 and 15 days after induced 2 (98 and 34 kDa) novel proteins. Samples collected from 30 DAI expressed 7 (30, 27,

Table 28. Changes in peroxidase activity in response to PRSV inoculation

Varieties	Treatments	*Peroxidase activity (changes in absorbance min^{-1} g^{-1} fresh weight)				
		Days after inoculation				
		5	10	15	30	60
Pusa Nanha	Uninoculated	9.51[c]	14.39[b]	5.36[d]	3.30[b]	1.41[c]
	Inoculated	9.62[c]	20.42[a]	11.37[a]	6.99[a]	3.21[a]
Local variety	Uninoculated	25.29[b]	8.98[d]	6.25[c]	3.32[b]	2.69[b]
	Inoculated	25.90[a]	11.71[c]	7.51[b]	3.35[b]	2.71[b]
CD (0.05)		0.579	0.189	0.213	0.292	0.017

*Mean of three replications

Table 29. Changes in polyphenol oxidase activity in response to PRSV inoculation

Varieties	Treatments	*Polyphenol oxidase activity (changes in absorbance min^{-1} g^{-1} fresh weight)				
		Days after inoculation				
		5	10	15	30	60
Pusa Nanha	Uninoculated	0.60[a]	0.50[a]	0.06[c]	0.11[b]	0.11[b]
	Inoculated	0.06[c]	0.50[a]	0.15[a]	0.21[a]	0.21[a]
Local variety	Uninoculated	0.31[b]	0.20[b]	0.11[b]	0.11[b]	0.11[b]
	Inoculated	0.31[b]	0.21[b]	0.11[b]	0.11[b]	0.11[b]
CD (0.05)		0.027	0.008	0.014	0.018	0.015

*Mean of replications

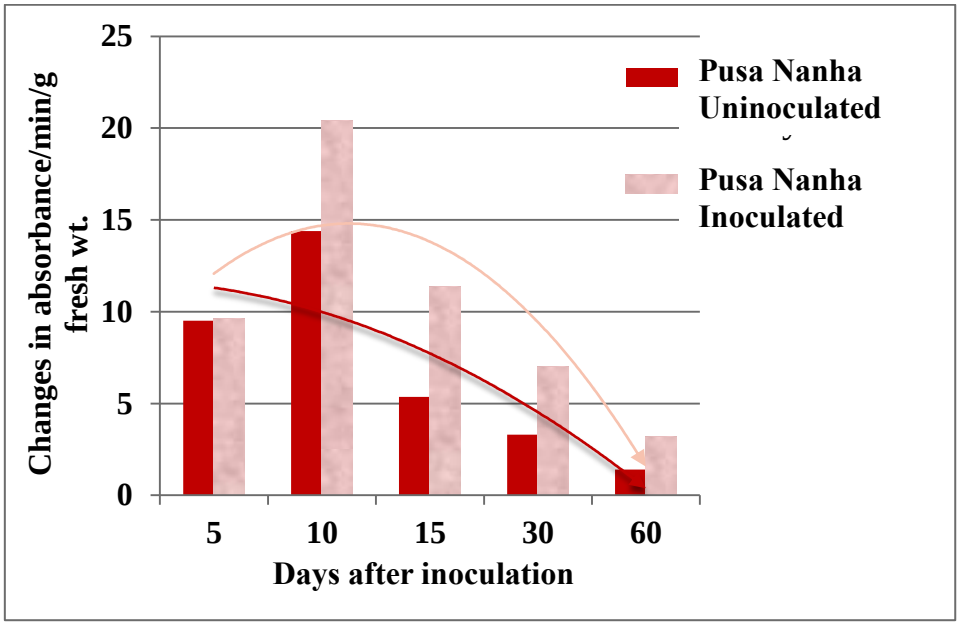

Fig 18a. Pusa Nanha

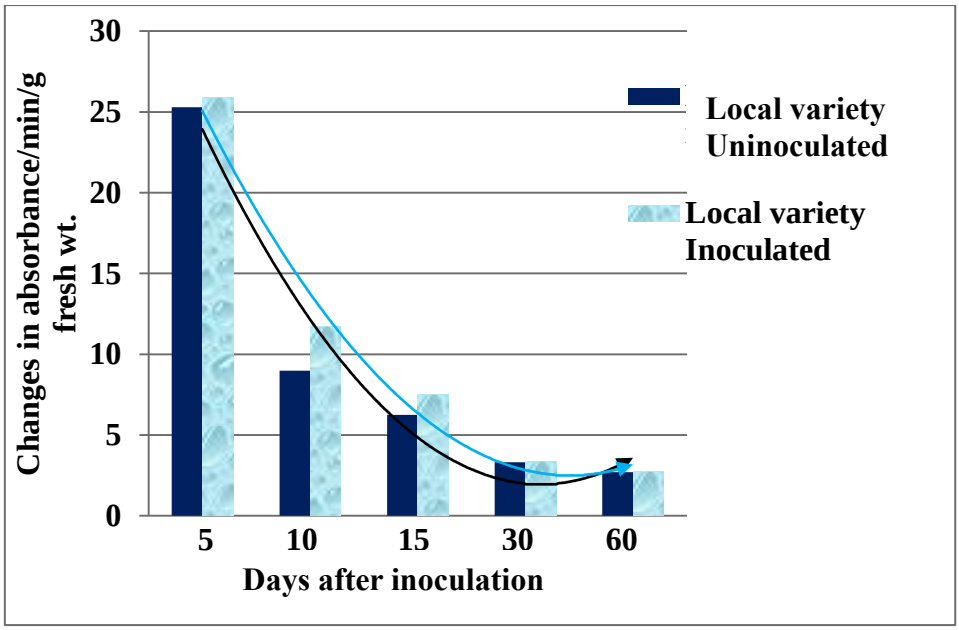

Fig 18b. Local variety

Changes in peroxidase activity in response to PRSV inoculation

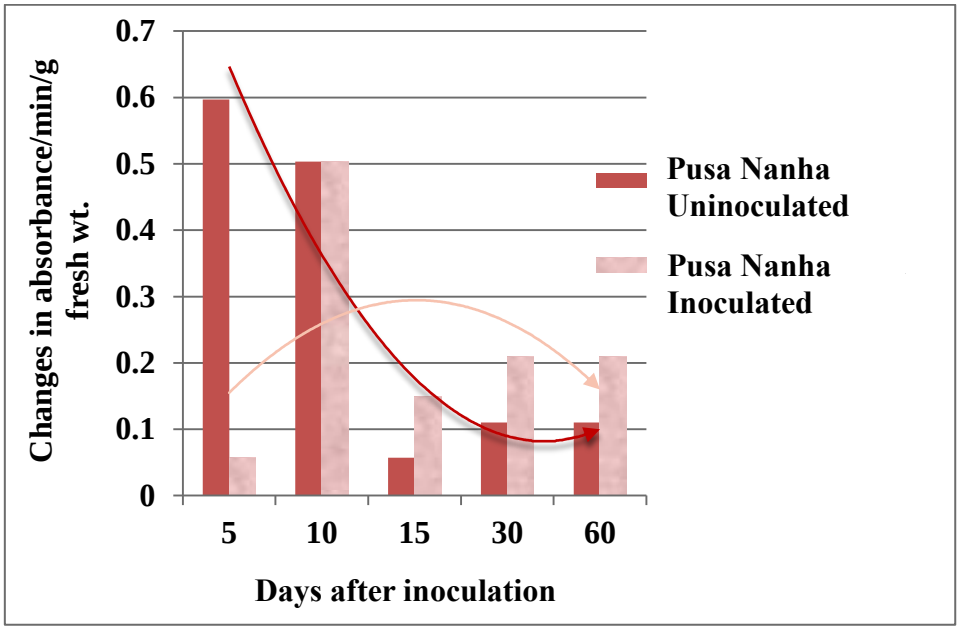

Fig 19a. Pusa Nanha

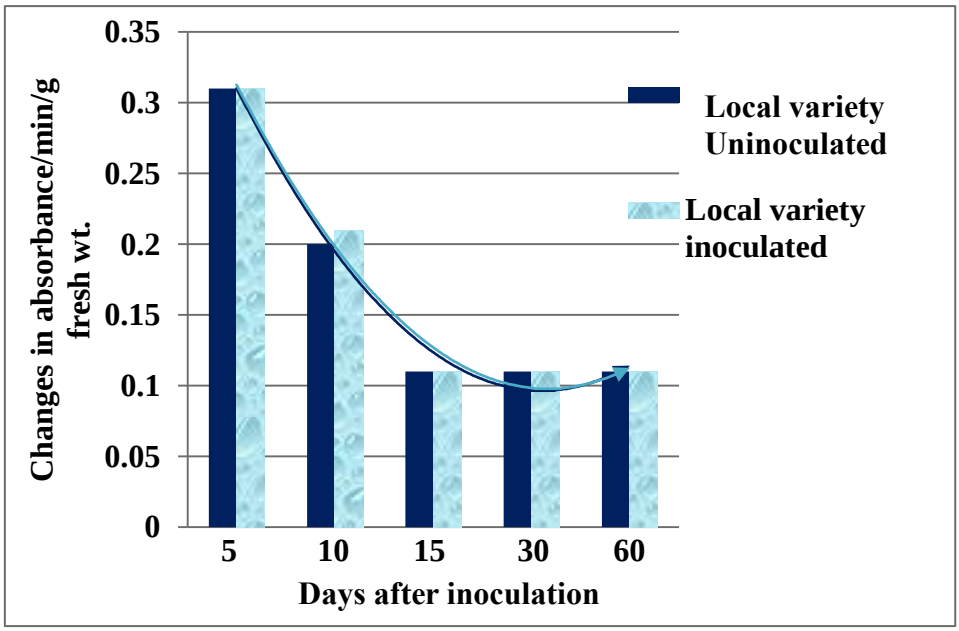

Fig 19b. Local variety

Changes polyphenol oxidase activity in response to PRSV inoculation

Table 30. Changes in phenylalanine ammonialyase activity in response to PRSV inoculation

Varieties	Treatments	*Phenylalanine ammonialyase activity ($\mu g\ g^{-1}\ min^{-1}$ fresh weight)				
		Days after inoculation				
		5	10	15	30	60
Pusa Nanha	Uninoculated	75.72[d]	58.04[c]	69.41[b]	59.38[b]	64.93[b]
	Inoculated	98.02[b]	105.90[a]	106.10[a]	68.02[a]	75.44[a]
Local variety	Uninoculated	99.10[a]	70.41[b]	68.74[c]	53.31[c]	50.07[c]
	Inoculated	76.64[c]	57.84[d]	69.41[b]	52.23[d]	49.20[d]
CD (0.05)		0.098	0.071	0.096	0.510	0.068

*Mean of replications

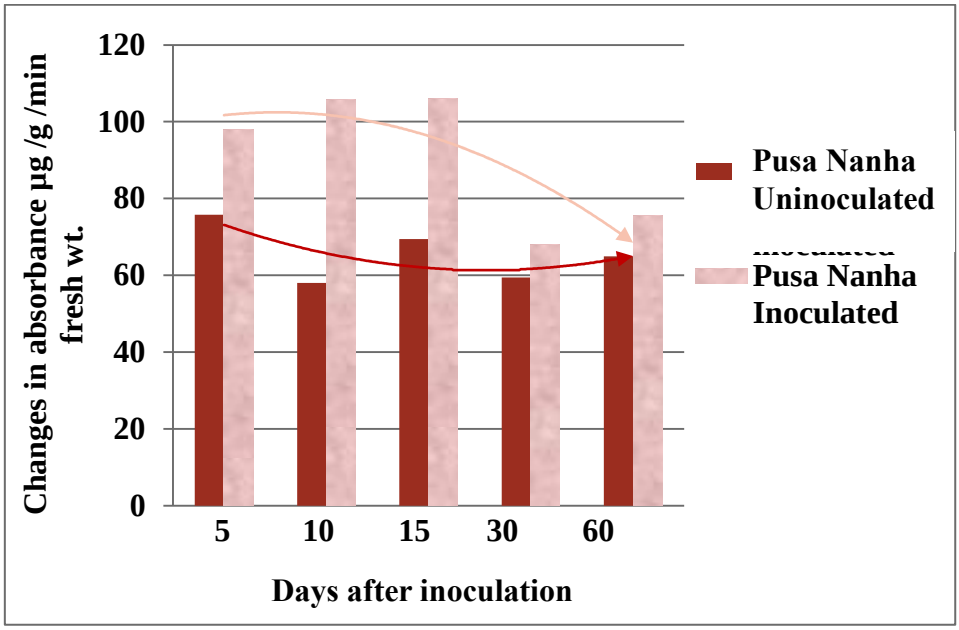

Fig 20a. Pusa Nanha

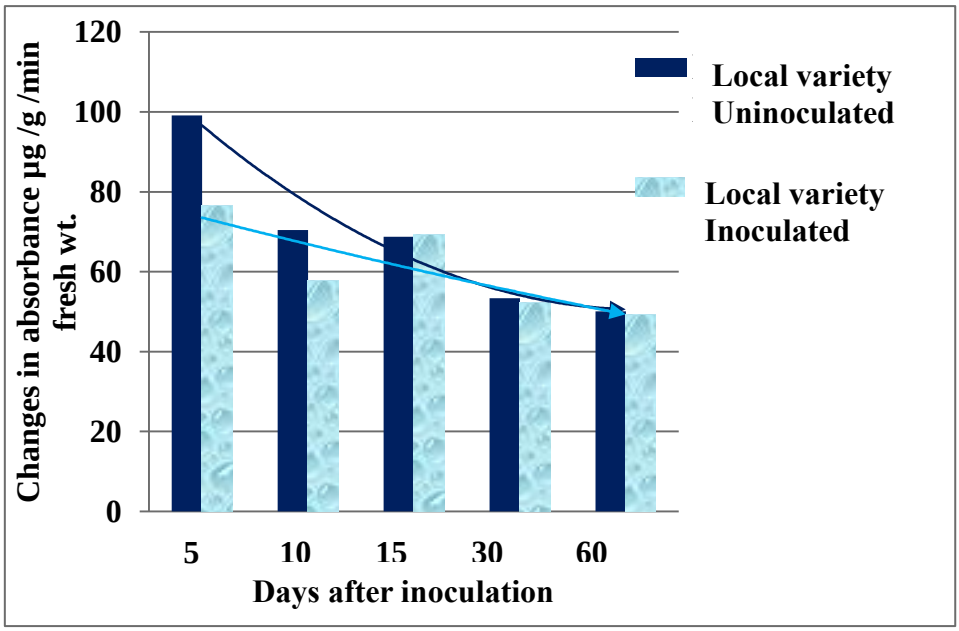

Fig 20b. Local variety

Changes in phenylalanine ammonialyase activity in response to PRSV inoculation

25, 18, 15, 12 and 9 kDa) additional novel proteins when compared to other treatments.

Protein profiles studies of uninoculated and inoculated papaya plants of local variety were analysed and found 5 new proteins were expressed in uninoculated healthy plants (Plate 13b). In inoculated plants, most of proteins were also expressed similar to that of healthy. Plants inoculated at 10 and 15 days after induced 2 (34 and 10 kDa) novel proteins. Samples collected from 30 DAI expressed 6 (30, 29, 27, 24, 16 and 14 kDa) additional novel proteins when compared to other treatments.

4.2.3.7 Electrophoretic Analysis of Isozyme

In the uninoculated plants of Pusa Nanha, 2 isozymes were detected (a and b) with Rm values of 0.03 and 0.08 respectively (Plate 14a and Table 31). In addition to the two isozymes, Pusa Nanha exhibited 2 more isozymes (c and d) at various stages of inoculation. The isozyme 'c' (Rm value of 0.23) was identified at 5, 10, 15 and 30 DAI. Isozyme 'd' (Rm value of 0.14) was detected at 60 DAI.

In the uninoculated plants of local variety, one isozyme 'a' (Rm value of 0.03) was detected (Plate 14b and Table 32). In addition local variety exhibited 2 more isozymes (b and c) at various stages of inoculation. Isozyme 'b' (Rm value of 0.11) was detected throughout the period of observation. Isozyme 'c' (Rm value of 0.14) was detected at 60 DAI. Intensity of 'a' isozyme diminished over the days of inoculation.

4.2.4 BIOLOGICAL DETECTION

4.2.4.1 Indicator Hosts for Diagnosis

Attempts were made to mechanically inoculate PRSV to *Chenopodium amaranticolor* and *Chenopodium quinoa.* Mechanical inoculation of PRSV to *C. amaranticolor* did not produce any symptom, whereas inoculation resulted in the development of numerous chlorotic local lesions on *C. quinoa* (Plate 15). Later the lesions turned necrotic. Thus *C. quinoa* was identified as the indicator plant for the biological detection of PRSV.

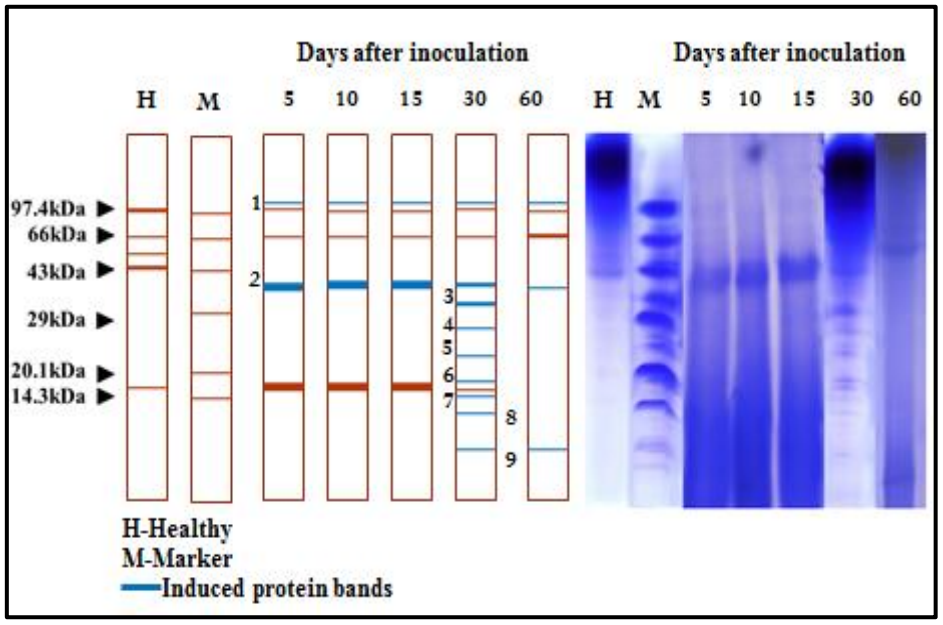

Plate 13a. Pusa Nanha

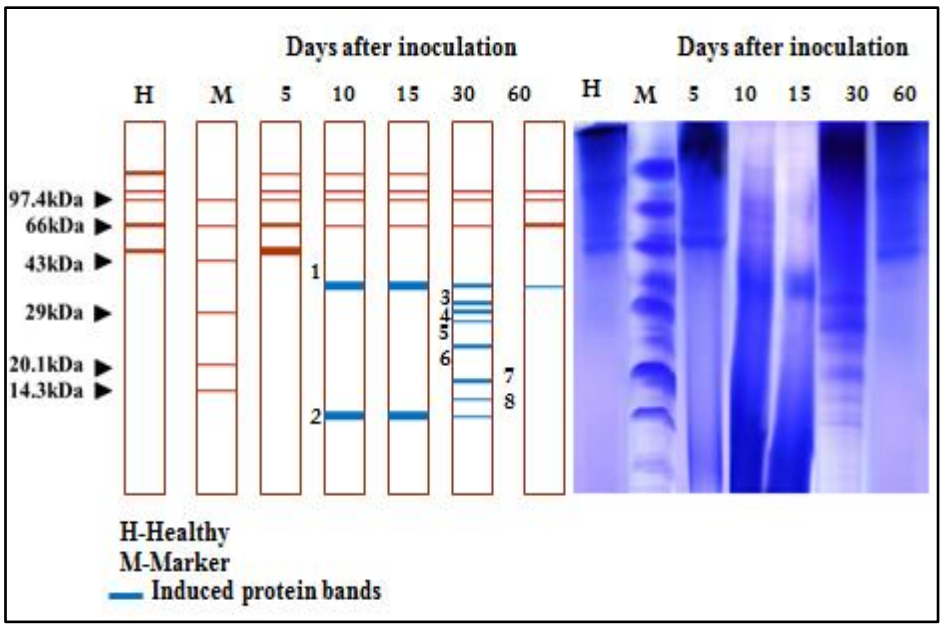

Plate 13b. Local variety of papaya

Protein profile in response to PRSV inoculation

Table 31. Isoperoxidase activity of Pusa Nanha in response to PRSV inoculation

Treatments	Isoperoxidase activity (*Rm value)			
	Iso PO1 (a)	Iso PO2 (b)	Iso PO3 (c)	Iso PO4 (d)
Uninoculated control	0.03	0.08	-	-
5 DAI	0.03	0.08	0.23	-
10 DAI	0.03	0.08	0.23	-
15 DAI	0.03	0.08	0.23	-
30 DAI	0.03	0.08	0.23	-
60 DAI	0.03	0.08	-	0.14

*Relative mobility

Table 32. Isoperoxidase activity of papaya local variety in response to PRSV inoculation

Treatments	Isoperoxidase activity (*Rm value)		
	Iso PO1 (a)	Iso PO2 (b)	Iso PO3 (c)
Uninoculated control	0.03	-	-
5 DAI	0.03	0.11	-
10 DAI	0.03	0.11	-
15 DAI	0.03	0.11	-
30 DAI	0.03	0.11	-
60 DAI	0.03	0.11	0.14

*Relative mobility

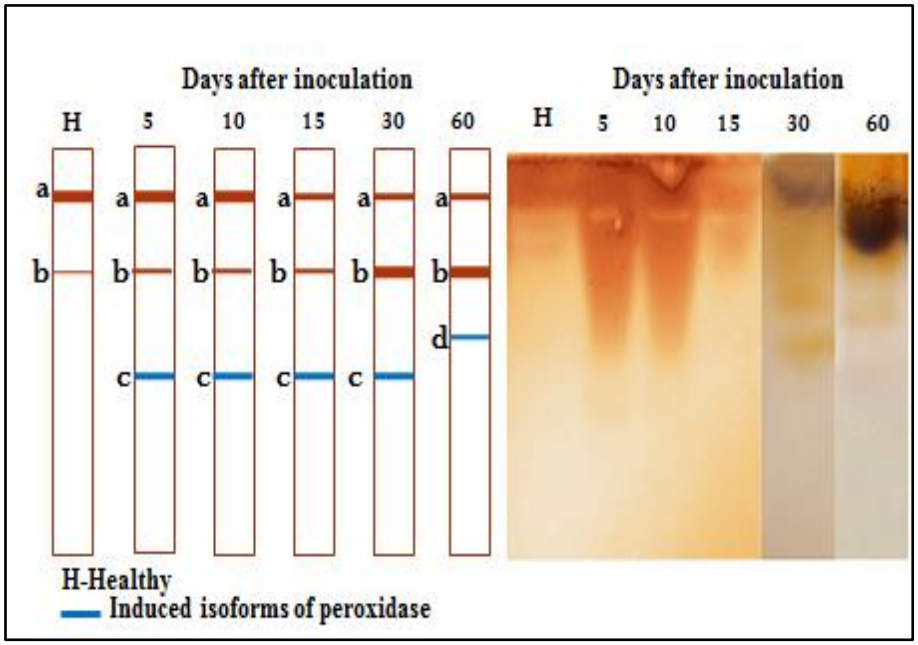

Plate 14a. Pusa Nanha

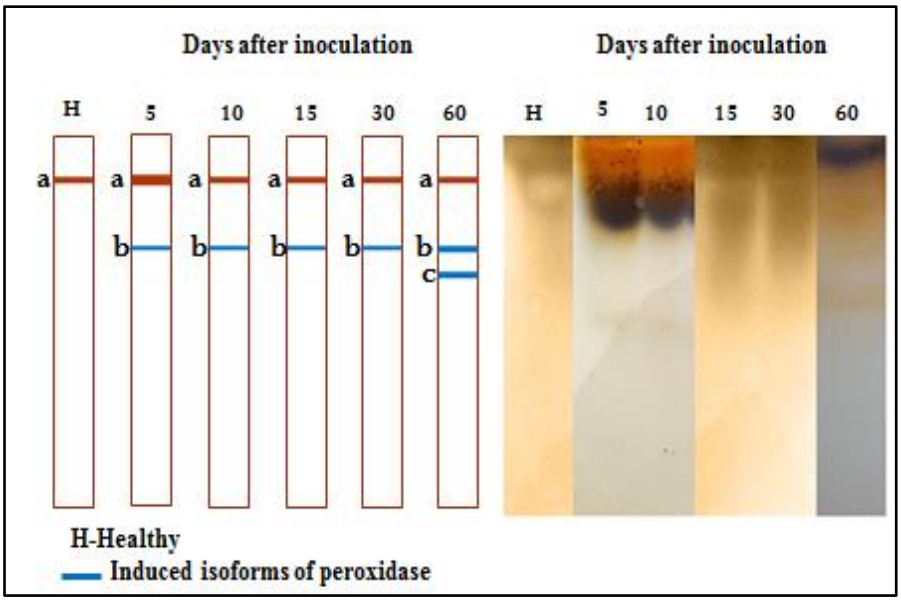

Plate 14b. Local variety of papaya

Isoperoxidase profile in response to PRSV inoculation

4.2.5 IMMUNOLOGICAL STUDIES

4.2.5.1 Direct antigen coating-Enzyme linked immunosorbent assay (DAC-ELISA)

DAC-ELISA was used to detect the virus causing ring spot disease of papaya in Kerala. The typical symptoms *viz*. mosaic, ring spot and shoestring symptoms were separately analysed to quantify the variations in virus inoculum showing different symptoms, based on their absorbance values. Results are presented in Table 33. DAC-ELISA was also used to detect the presence of PRSV in papaya mealy bug, using the polyclonal antibody against PRSV. Results are shown in Table 34.

Samples collected from papaya infected with the virus gave positive reaction to the polyclonal antibody against PRSV and *Potyvirus* group antiserum (Plate 16). Based on the observed absorbance values, variations in virus inoculum in the different symptoms were graded as mosaic> shoestring> ring spot. The Optical Density (OD) values from the extracts of mealy bugs after acquisition feeding on infected papaya seedling also gave positive reaction when treated with polyclonal antiserum against PRSV (Plate 11b).

4.2.5.2 Dot immunobinding assay (DIBA)

DIBA was conducted to detect the presence of the virus causing ring spot disease in papaya (Plate 17). PRSV was detected in the crude sap collected from the infected plant samples. The infected leaf samples showed purple coloured spots on nitrocellulose membrane indicating positive reaction unlike those produced by the healthy ones and buffer check which gave a colourless reaction**.**

4.2.6 MOLECULAR DETECTION AND CHARACTERISATION

4.2.6.1 Reverse transcription-Polymerase chain reaction (RT-PCR)

Molecular diagnosis using RT-PCR followed by gel electrophoresis was performed for the specific detection of the virus causing ring spot disease in

Table 33. Reaction of polyclonal antibodies against PRSV showing different symptoms

Polyclonal antibodies	Symptoms tested	*Absorbance at 405 nm		Reaction
		Healthy	Infected	
PRSV	Mosaic	0.050	1.307	+
	Ring spot	0.040	1.183	+
	Shoestring	0.044	1.241	+
Poty	Mosaic	0.023	0.097	+
	Ring spot	0.003	0.027	+
	Shoestring	0.007	0.041	+

*Mean of three replications

Table 34. Reaction of papaya mealy bug associated with infected papaya plants

Polyclonal antibody	*Absorbance at 405 nm		Reaction
	Control	Infected	
PRSV	0.045	1.244	+

*Mean of three replications

+ Presence of virus
- Absence of virus

Plate 15. *Chenopodium quinoa* on inoculation with PRSV

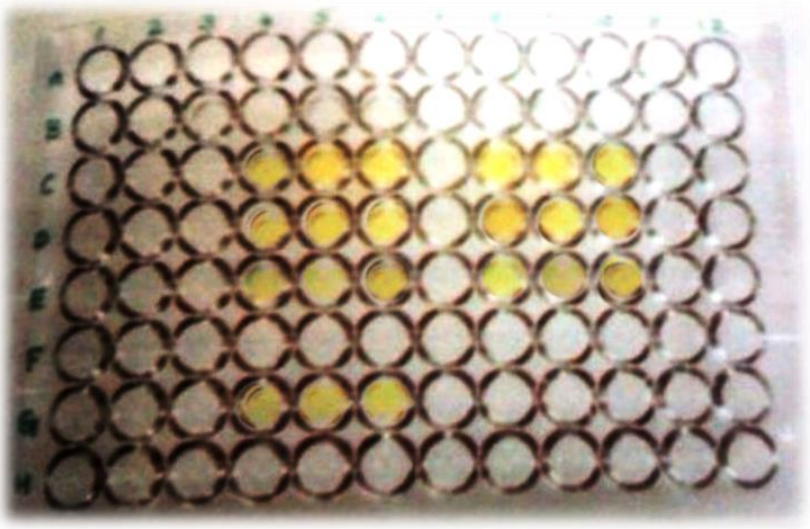

Plate 16. Reaction of PRSV in DAC-ELISA

Positive reaction to *Potyvirus* group- D8, D9 and D10; E4, E5 and E6; E8, E9 and E10

Positive reaction to PRSV- C4, C5 and C6; C8, C9 and C10; D4, D5 and D6

Positive reaction to papaya mealy bug- G4, G5 and G6

D **B** **H**

D: Diseased **B: Buffer** **H: Healthy**

Plate 17. Positive reaction of polyclonal antibody against PRSV in DIBA

papaya. Primers specifically designed based on the nucleotide sequence of coat protein (CP) region of the PRSV was used for the amplification of CP gene. An amplicon of size 220 bp (Plate 18c), was obtained from the c-DNA reverse transcribed (Plate 18b), from the viral RNA isolated (Plate 18a), from the infected papaya leaf sample using the PRSV specific primer. RT-PCR results confirmed that, the ring spot disease in papaya in Kerala was caused by the *Papaya ring spot virus* (PRSV).

4.2.6.2 Characterisation

The PCR product obtained was sequenced and sequence was analysed using BLAST software (Fig 21 and Table 35). Comparative nucleotide sequence alignment of the virus with the available data bases from NCBI revealed a 99 per cent homology with PRSV isolate from Andhra Pradesh. Isolate also shared 93 per cent homology with PRSV isolate biotype W from Brazil. Comparative amino acid sequence alignment of the isolate revealed a 100 per cent homology with coat proteins of PRSV (Fig 22).

Comparative sequence analysis of BlCMV and PRSV *Potyviruses* (using Clustal Omega software) showed that the sequences were entirely different and showed only a 51.12 per cent similarity (Fig 23) at nucleotide levels.

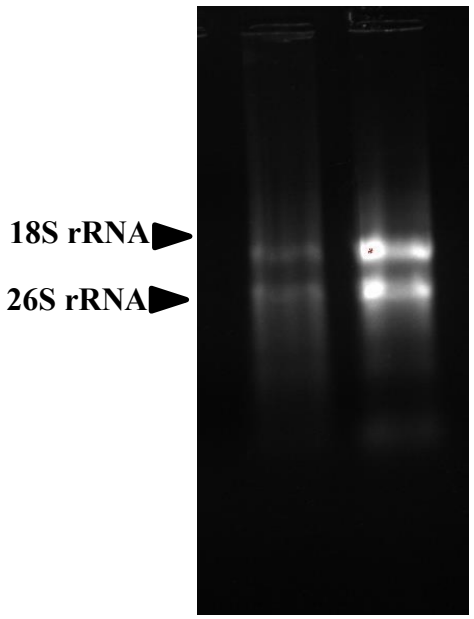

Plate 18a. Total RNA

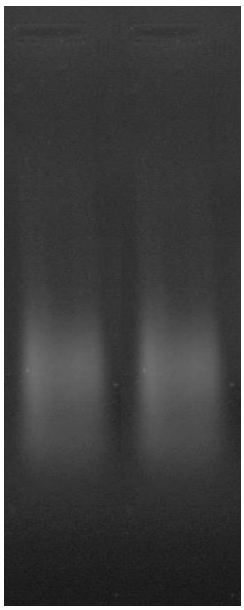

Plate 18b. cDNA

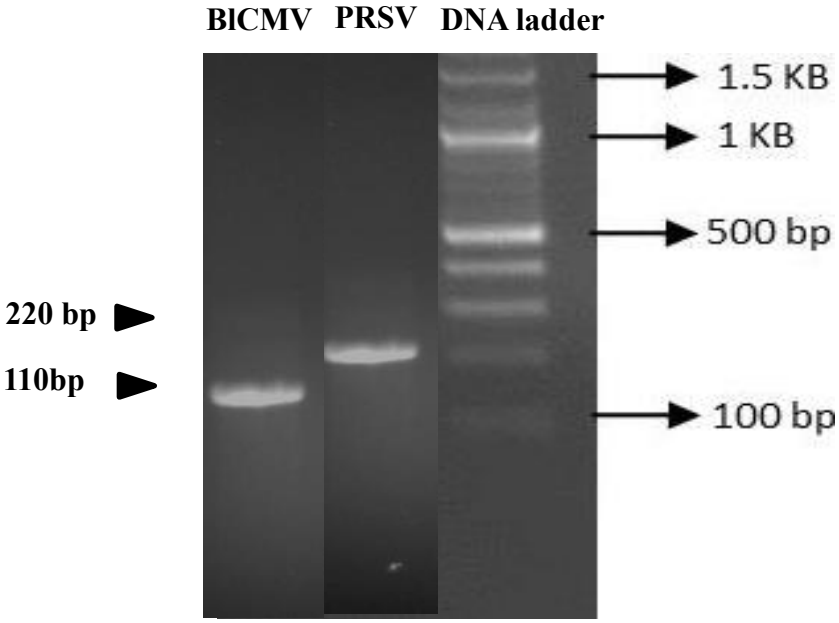

Plate 18c. RT-PCR Amplification

Molecular diagnosis of PRSV

Table 35. Comparative nucleotide sequence alignment of PRSV isolate with reported isolates of *Potyviruses*

Accession No.	Description	Nucleotide identity (%)
DQ666640.1	*Papaya ring spot virus* isolate PRSV-KE Ca polyprotein gene, partial cds	99
AY238884.1	*Papaya ring spot virus* isolate B coat protein gene, partial cds	98
DQ666638.1	*Papaya ring spot virus* isolate PRSV-AP Ko polyprotein gene, partial cds	98
HM626464.1	*Papaya ring spot virus* isolate Coimbatore TNAU orchard polyprotein gene, partial cds	97
HM626467.1	*Papaya ring spot virus* isolate Dharapuram polyprotein gene, partial cds	96
DQ192587.1	*Papaya ring spot virus* isolate P polyprotein mRNA, partial cds	96
AY458618.1	*Papaya ring spot virus* isolate Karnataka Dharwad coat protein gene, partial cds	95
KC149501.1	*Papaya ring spot virus* P isolate MP coat protein gene, partial cds	95
AF344647.1/AF344647	*Papaya ring spot virus* isolate CE biotype P coat protein (cp) gene, partial cds	93
JN132468.1	*Papaya ring spot virus* W isolate T13 polyprotein gene, partial cds	92

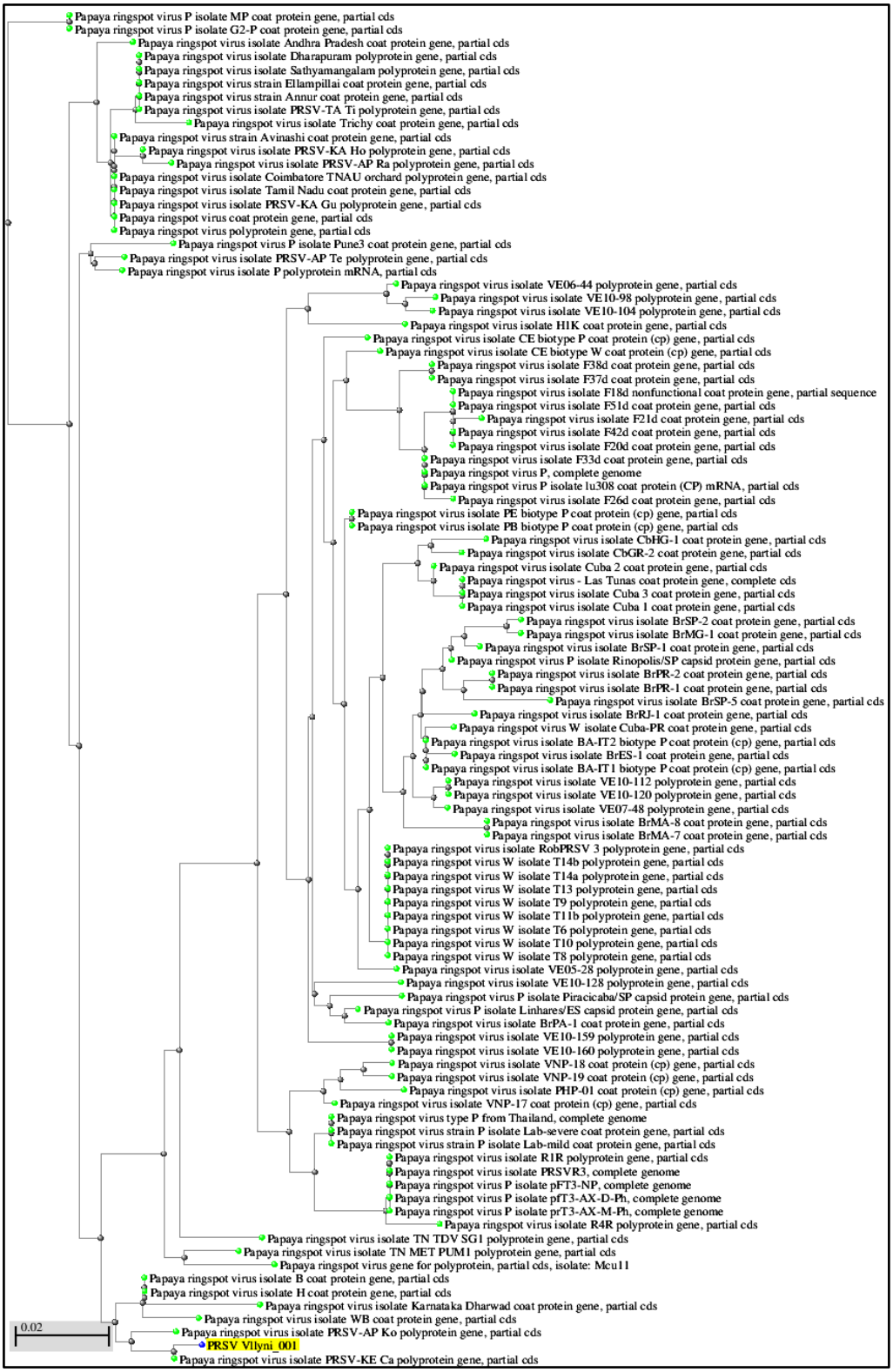

Fig 21. Dendrogram showing the relationship of Vellayani isolate of PRSV with related *Potyviruses*

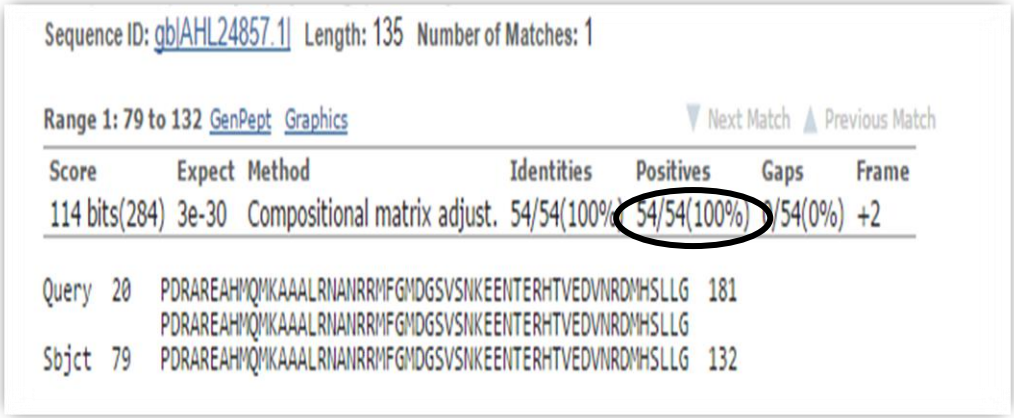

Fig 22. Comparative amino acid sequence alignment of Vellayani isolate of PRSV with its coat protein sequence

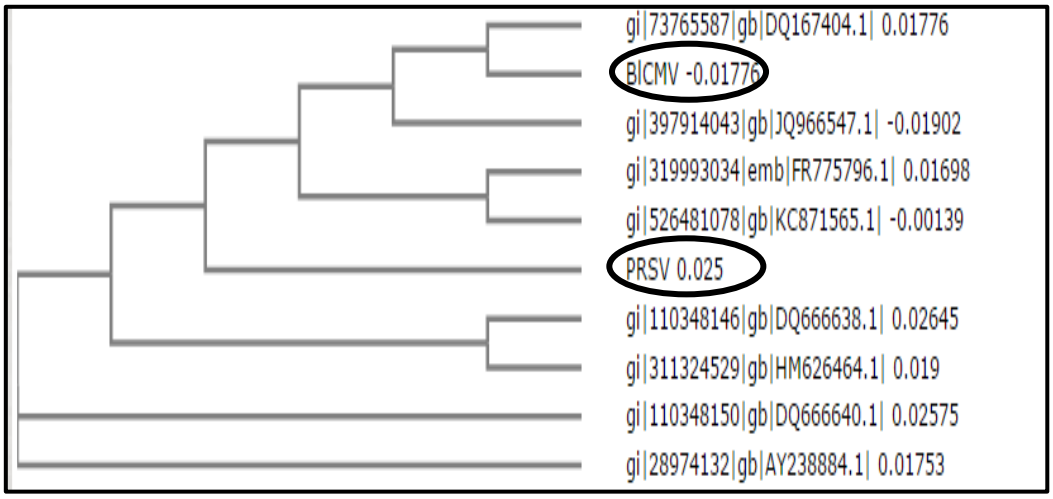

Fig 23a. Dendrogram showing the relationship of BlCMV and PRSV

1:	gi|73765587|gb|DQ167404.1|	100.00	-nan	46.26	48.60	43.78	48.60	48.36	-nan	51.14	-nan
2:	PRSV	-nan	100.00	89.01	89.01	90.66	89.56	57.46	51.52	57.96	59.72
3:	gi|110348146|gb|DQ666638.1|	46.26	89.01	100.00	95.45	93.86	94.97	59.33	52.27	60.68	55.56
4:	gi|311324529|gb|HM626464.1|	48.60	89.01	95.45	100.00	94.52	95.20	60.43	51.52	65.15	59.87
5:	gi|110348150|gb|DQ666640.1|	43.78	90.66	93.86	94.52	100.00	95.67	58.40	51.52	60.32	55.56
6:	gi|28974132|gb|AY238884.1|	48.60	89.56	94.97	95.20	95.67	100.00	60.05	50.76	64.01	58.55
7:	gi|319993034|emb|FR775796.1|	48.36	57.46	59.33	60.43	58.40	60.05	100.00	86.36	98.44	99.38
8:	BlCMV	-nan	51.52	52.27	51.52	51.52	50.76	86.36	100.00	85.61	85.61
9:	gi|526481078|gb|KC871565.1|	51.14	57.46	60.68	65.15	60.32	64.01	98.44	85.61	100.00	96.52
10:	gi|397914043|gb|JQ966547.1|	-nan	59.72	55.56	59.87	55.56	58.55	99.38	85.61	96.52	100.00

Fig 23b. Percentage identity matrix

Comparative nucleotide sequence alignment of Vellayani isolates of BlCMV and PRSV

Discussion

5. DISCUSSION

The results of the study on the symptomatology and transmission of *Potyviruses* infecting cowpea and papaya, along with their biological, immunological, molecular detection and characterisation are discussed in this chapter.

Cowpea (*Vigna unguiculata* (L.) Walp.) Family Leguminosae, an important dual purpose legume crop and papaya (*Carica papaya* L), Family Caricaceae, an important fruit crop, widely grown in tropics and subtropics, are severely affected by the *Potyvirus* genus, the most economically devastating genera among plant viruses. The *Potyviridae* genome, which is 8500 to 10000 nucleotides long, consists of either one molecule or two segments of linear positive-sense single stranded RNA. The *Potyviruses* taken for the study were *Blackeye cowpea mosaic virus* (BlCMV) causing mosaic disease in cowpea and *Papaya ring spot virus* (PRSV) causing ring spot disease in papaya, owing to their widespread occurrence among the cultivars in Kerala.

Taking into consideration the destructive losses caused by the *Potyviruses*, symptomatology and transmission of *Potyviruses* infecting cowpea and papaya, along with their biological, immunological, molecular detection and characterisation were conducted.

5.1. *Blackeye cowpea mosaic virus* (BlCMV)

5.1.1. SYMPTOMATOLOGY

The results revealed that majority of plants of susceptible cultivar, Vellayani Jyothika and a very few plants of resistant cultivar, CO6, developed the initial symptoms of vein clearing 7 days after inoculation (DAI) with BlCMV. However, delayed symptom expression (10-12 DAI) and rapid loss in disease severity was noticed in the resistant genotype. Similar observations of delayed symptom expression followed by symptom remission in resistant cultivars were reported by other workers in different plant-virus systems (Anderson *et al*., 1989;

Akthar *et al*., 2013; Siddique *et al*., 2014). Vellayani Jyothika on inoculation with BlCMV resulted in a wide range of symptoms including dark green and light green mottling, mosaic and vein banding on leaves, reduction in leaf size and distortion, accompanied with severe stunting of plants. The plants also showed severe stunting and resulted in reduction in number of flowers and pods. Similar symptoms were described by Bock and Conti (1974) as dark green vein banding, leaf distortion, blistering and stunting of BlCMV infected cowpea plants. Bashir *et al*. (2002) have also observed the appearance of symptoms comprising dark vein banding and interveinal chlorosis on cowpea leaves.

Contradictory to the present observations, Thottappilly and Rossel (1985) have reported that B1CMV produced localized symptoms on cowpea which were large reddish, often ring-like lesions which typically spread along the veins, forming a reddish-net pattern. The variations in the symptoms could be due to the type of viral strains infecting the plant, cowpea cultivar, the time of infection of the virus (time of the year and stage of plant growth), light intensity, environmental temperature, mixed infections and/or presence of yet unidentified pathogens, as suggested by Jones *et al.* (1991).

5.1.2. TRANSMISSION STUDIES

Transmission studies of BlCMV were conducted *viz.* through sap, seeds and insect vectors.

A transmission of 80 per cent was observed for BlCMV through mechanical inoculation. Successful sap transmission of BlCMV and related strains were reported by many workers (Murphy *et al.*, 1987; Damiri *et al.*, 2012). Ndiaye *et al*. (1993); Elkewey *et al*. (2007) reported mechanical transmission of 100 and 90 per cent respectively for *Cowpea aphid-borne mosaic virus* (CABMV) in cowpea, which is in conformity with the present study.

The present study also revealed the seed borne nature of BlCMV, with a seed transmission of 56 per cent. A similar observation was made by Bashir (1992), who found that 21 accessions were infected either with B1CMV (0.0 to

6.9 per cent) or CABMV (0.0 to 13.3 per cent), out of the 158 *Vigna unguiculata* plant introductions and germplasm accessions tested.

Insect transmission studies revealed that, *Aphis craccivora* Koch and *Aphis gossypii* Glover were equally efficient vectors of BlCMV, with a recorded transmission of 30 per cent. This agrees with the findings of Bashir and Hampton (1994) who reported a non-persistent aphid transmission for BlCMV, using three aphids (*A.craccivora*) per cowpea plant. Similarly, Attiri *et al.* (1986) reported transmission of CABMV to and from cowpea, by three species in the genus *Aphis viz. A. craccivora, A. spiraecola* and *A. gossypii.*

5.1.3. HOST PATHOGEN INTERACTION STUDIES

Resistant varieties form an integral part of management of viral disease of crop plants. But unfortunately host plant resistance was rapidly overcome by the frequent change in strains of *Potyviruses.* In the present investigation, attempt was made to know the biochemical basis of resistance to BlCMV in resistant and susceptible cultivars of cowpea. Biochemical changes *viz.* carbohydrate, chlorophyll, phenol, protein contents, activities of defense related enzymes, protein profile studies and isozyme analysis in compatible (Vellayani Jyothika) and incompatible (CO6) host pathogen interactions were studied.

5.1.3.1. Estimation of Total Carbohydrate

Total carbohydrate content was found to differ non-significantly in the inoculated plants of CO6 (15.06 mg g^{-1} at 5 DAI to 14.00 mg g^{-1} at 60 DAI) compared to the uninoculated plants. However, Vellayani Jyothika showed a significant decrease (8.17 mg g^{-1} at 10 DAI to 10.09 mg g^{-1} at 60 DAI), compared to the uninoculated plants beyond 5 DAI. Concurrent to the present findings, Mali *et al*. (2000) reported that *Mungbean yellow mosaic virus* (MYMV) infection on moth bean (*Vigna aconitifolia*) resulted in significant reduction of total soluble carbohydrates in susceptible cultivar (GMO 9101) when compared to resistant cultivar (CZM 79). The decrease in carbohydrate content can be explained on the

basis of faster breakdown of carbohydrates due to increased respiration, followed by conversion of carbohydrates into amino acids (Sinha and Srivastava, 2010).

5.1.3.2. Estimation of Chlorophyll

Chlorophyll a, chlorophyll b and total chlorophyll contents changed non-significantly in inoculated plants of CO6 at 10 (1.18, 0.41 and 1.59 mg g^{-1}), 15 (1.42, 0.45 and 1.87 mg g^{-1}), 30 (1.96, 0.42 and 2.37 mg g^{-1}) and 60 DAI (2.10, 0.46 and 2.56 mg g^{-1} mg g^{-1}) compared to the uninoculated plants. Chlorophyll a, chlorophyl b and total chlorophyll contents of inoculated susceptible plants decreased significantly from the uninoculated control plants. The results were similar to other compatible and incompatible host pathogen interaction studies (Ashraf *et al*., 2000; Lopes and Berger, 2001).

Radhika and Umamaheswaran (2000) also reported a higher chlorophyll content in resistant variety, CO6 compared to susceptible variety, Sharika on inoculation with BlCMV. The present observation was in concurrence with the reduced chlorophyll content in mothbean (*Vigna aconitifolia*) (Arora *et al*., 2009) and mungbean (*Phaseolus aureus*) infected with MYMV (Sinha and Srivastava, 2010). Ahmed *et al.* (1986) found that the reduction in chlorophyll content might be due to the increased chlorophyllase activity of virus infected plants. Zhou *et al.* (2004); Guo *et al.* (2005) opined that the inhibition of enzymes associated with carbon assimilation is yet another reason for the reduced chlorophyll content. Reduction in chlorophyll content due to *Potyvirus* infection was caused by the aggregation and consequent reduction in the chloroplast number (Jin *et al*., 2007; Huang *et al*., 2010; Wei *et al.*, 2013).

5.1.3.3. Estimation of Phenol

Phenolic compounds enhance the mechanical strength of host cell walls by the synthesis of lignin and suberin. These were in turn involved in the formation of physical barriers thus blocking the spread of pathogens (Singh *et al.*, 2010; Ngadze *et al*., 2012). The results showed a significant increase in the phenol content of the inoculated plants of resistant genotypes (1.46 mg g^{-1} at 5 DAI to

12.27 mg g^{-1} at 60 DAI), compared to the susceptible genotype. However, Vellayani Jyothika recorded a constant value (4.23 mg g^{-1}) at 30 and 60 DAI.

In line with the findings of present study, Dantre (1983); Singh and Srivastav (1999); Shilpasree *et al.* (2013) have reported that BlCMV infection increased the total phenols in diseased cowpea compared to healthy ones. Increased level of phenols suggested an acceleration of phenols synthesizing pathway following pathogen infection (Meena *et al.,* 2008).

5.1.3.4. Estimation of Total Soluble Protein

A significant increase in protein content was noted in the inoculated plants of CO6 (1.53 mg g^{-1} at 5 DAI to 6.61 mg g^{-1} at 60 DAI), whereas no significant difference was observed in susceptible plants at 30 (2.90 mg g^{-1}) and 60 DAI (2.50 mg g^{-1}). An increase in protein level in virus infected plants have also been reported by several workers (Chakraborty *et al*., 1993; Shivaprasad *et al*., 2005; Hofius *et al*., 2001; Szczepanski and Redolfi, 2008). Due to virus infection, there was an increased demand for abnormal protein production which was required for the rapid synthesis of virus particles. This demand was met upon by the increased diversion of assimilated carbon compounds towards protein synthesis (Sinha and Srivastava, 2010)**.**

5.1.3.5. Estimation of Defense Related Enzymes

Early and elevated levels of expression of various defense related enzymes are an important feature of host plant resistance to pathogens (Vanitha & Umesha 2008).

Peroxidase (PO) is a well known class of Pathogenesis related proteins (PR proteins) and is expressed to limit cellular spreading of infection through establishment of structural barriers or generation of reactive oxygen species and reactive nitrogen species (Passardi *et al.,* 2004). Phenylalanine ammonialyase (PAL) is one of the key defense enzyme involved in phenyl propanoid biosynthesis (Hemm *et al*., 2004). The study revealed a significant increase in the PO and PAL activity in the inoculated plants of resistant genotype, compared to

the susceptible genotype with maximum activities recorded at 30 DAI (81.53 mg g^{-1}) and 5 DAI (80.32 mg g^{-1}) respectively. The present findings were in concurrence with the observations made by Mydlarz and Harvell (2006); Aboshosha *et al*. (2008).

Polyphenol oxidases (PPOs) are enzymes, belonging to a group of copper containing metallo proteins and are members of oxido reductases, that catalyse the oxidation of a wide range of phenolic compounds by utilizing molecular oxygen (Queiroz *et al*., 2008). Resistant genotype on inoculation, exhibited a significant increase in PPO activity at 5 DAI (1.61 min^{-1} g^{-1}) and 60 DAI (4.27 min^{-1} g^{-1}). However the susceptible genotype recorded the maximum enzyme activity at 5 DAI (1.22 min^{-1} g^{-1}) and slowly declined thereafter. Radhika and Umamaheswaran (2000) also reported a higher activity of PO, PPO and PAL in resistant variety of cowpea, CO6 when compared to susceptible variety, Sharika.

Total soluble phenols together with PPO appear to play a role in resistance to BlCMV as resistant genotype produced more secondary metabolites involved in plant defense mechanisms than the susceptible genotype. The present observation was supported by the work of Kumar *et al.* (1991); Li and Steffens (2002), Ngadze *et al.* (2012). The present study also indicated that activation of PAL and subsequent increase in phenol content was a general response associated with BlCMV resistance. The observation was in tune with those made by Siddique *et al.* (2014) who reported an activation of PAL in resistant cotton genotypes, Ravi and Co Tiep Khac (21.1 and 12.86 per cent respectively) and subsequent increase in phenol content (13.48 and 12.01 per cent respectively), in response to *Cotton leaf curl Burewala virus* (CLCuBuV) infection.

5.1.3.6. Electrophoretic Analysis of Proteins using SDS-PAGE

Protein profile studies of inoculated plants at 5, 10, 15, 30 and 60 DAI was conducted to elucidate the plant-virus interactions and consequent induction of novel proteins. The induced proteins play a crucial role in alleviating disease reactions in compatible and incompatible interactions. Expression of new

proteins was analyzed by Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). SDS-PAGE identified eleven and ten induced proteins in CO6 and Vellayani Jyothika respectively. The difference in protein pattern of the two genotypes might be due to their genetic differences. Specific proteins induced can be used as biochemical markers to identify resistance and susceptibility in plants toward BlCMV infection.

Similar to the present findings, resistance associated proteins were reported in several plant-virus interactions (Sela, 1981; Rathi *et al*., 1986). *Cucumber mosaic virus* (CMV) strain Y multiplication in resistant (Kurodane-Sanjaku) and susceptible (PI 189375) cowpea genotypes was measured by SDS-PAGE followed by electron blotting. PI 189375 accumulated considerable amounts of CP, both in inoculated and uninoculated plants. Viral CP was detected 1DAI, as a trace amount, which increased rapidly at 3DAI, then declined at 4DAI. However, CP was not detected in inoculated and uninoculated resistant genotype (Nasu *et al*., 1996). Acosta-Leal and Xiong (2008) performed SDS-PAGE on PVY infected tobacco cultivars and detected weak bands of Potato virus Y coat protein (PVY CP) in susceptible genotype protoplasts (Burley 21and NC745) at 48 h post-infection (hpi). The bands became stronger at 96 hpi. In contrast, the CP was detected in resistant genotype protoplast (VAM) only at 96 hpi, as a weak signal barely visible in the radiogram.

The additional proteins with molecular weight in the range of 34 kDa was assumed to be the capsid protein of BlCMV. The observation was in accordance with the findings of Zhao *et al*. (1991) who reported that purified isolate of BlCMV subjected to SDS-PAGE revealed a major and a minor protein component with relative molecular weights of 34.5 and 31 kDa respectively. Similarly, Bhadramurthy and Bhat (2009) reported that, the partially purified BCMV subjected to SDS-PAGE, revealed a major band corresponding to 34 kDa.

5.1.3.7. Electrophoretic Analysis of Isozyme

Isozyme analysis is a powerful tool for estimating genetic variability in identifying cultivars and germplasm accessions (Asiedu, 1992). Electrophoretic separation of isoperoxidases revealed that, isozymes with Relative mobility (Rm) values of 0.13, 0.20 and 0.28 were unique for the resistant genotype, CO6. Enhanced activity of isoperoxidases (a and b) of CO6, following infection with BlCMV, suggested activation of existing isoperoxidases. No unique bands were detected in the Vellayani Jyothika upon inoculation. There was no marked deviation from the control in the isozyme 'a' of Vellayani Jyothika, however increase in activity was recorded for isozyme 'b'.

Similar observations were also made by Umamaheswaran (1996) and Sindhu (2001). Shilpasree *et al.* (2013) have identified 3 isoperoxidases in resistant cowpea genotype, DCS-6 with Rm values of 0.46, 0.48 and 0.50. Also in the susceptible genotype, GC-3, 3 isoperoxidases were identified with Rm values of 0.65, 0.58 and 0.59.

5.1.4. BIOLOGICAL DETECTION

Chenopodium amaranticolor was identified as a potential indicator plant for the biological detection of BlCMV infection. However *Chenopodium quinoa* failed to develop any symptom on mechanical inoculation with BlCMV. Similar observations were made by other workers (Bock, 1973; Bhadramurthy and Bhat, 2009; Damiri *et al*., 2013). Also Lima *et al.* (1979) reported that BlCMV was readily transmitted from cowpea to *C. amaranticolor* and gave positive results. Contradictory to the present findings there are reports on the development of local lesions on *C. quinoa* (Anderson *et al*., 1989; Khatab and Eman, 2002; Ladipo *et al.,* 2004; Elkewey *et al.,* 2007).

5.1.5. IMMUNOLOGICAL STUDIES

Serological methods using polyclonal (PAb) and monoclonal (MAb) antibodies are efficient for the detection of BICMV (Hao *et al.,* 2003). The two

widely used serological assays *viz.* Direct antigen coating-Enzyme linked immunosorbent assay (DAC-ELISA) and Dot immunobinding assay (DIBA) were used for the present study.

DAC-ELISA using polyclonal (H2) and monoclonal (15E6 and 16G5) antibodies specific to BlCMV gave a positive reaction in samples collected from symptomatic plants. However, the virus infected cowpea plants did not reacted with the polyclonal and monoclonal antibodies specific to *Cowpea aphid-borne mosaic virus* (CABMV), confirming the virus causing mosaic disease in cowpea was BlCMV. In tune with the results of present study, Dijkstra *et al*. (1987) detected isolates of BICMV from germplasm of yard-long bean (*Vigna unguiculata* subsp. *sesquipedalis*) and soybean plants (*Glycine max*) using DAC-ELISA. Eighteen isolates of BCMV, five isolates of B1CMV, four isolates of CABMV, and one isolate each of *Adzuki bean mosaic virus* (AzMV), and *Peanut stripe virus* (PStV) were serologically detected and compared using a panel of 13 monoclonal antibodies, in ELISA (Mink and Silbernagel, 1992).

DIBA have been used for the identification of *Potyviruses* in cowpea plants (Sidaros *et al.*, 2006; El-Kewey *et al.*, 2007; Akinjogunla *et al.*, 2008). In the present study, DIBA was conducted, using polyclonal antiserum against BlCMV and CABMV *i.e.* H2 and H4 respectively. Only H2 gave positive colour reaction, thus identifying the virus as BlCMV. Koohapitagtam and Nualsri (2013) have also reported a specific reaction of BlCMV against polyclonal antiserum.

5.1.6. MOLECULAR DETECTION AND CHARACTERISATION

An amplicon of size 110 bp, was obtained from the viral RNA isolated from the infected cowpea leaf sample using the BlCMV specific primer. RT- PCR results revealed that, the mosaic disease of cowpea in Kerala was caused by BlCMV. Comparative nucleotide sequence alignment of the viruses with the available data bases from National Centre for Biotechnology Information (NCBI) revealed a 97 per cent homology with *Bean common mosaic virus* (BCMV) strain

Blackeye from Karnataka. Isolate also shared 93 and 92 per cent homology with two related *Potyviruses viz*. CABMV and *Peanut stripe virus* (PStV) respectively. Comparative amino acid sequence alignment revealed a 95 per cent homology with coat proteins of BCMV.

In line with the present findings, Vetten *et al*. (1992) reported on the serological relationships of Indian isolates of BlCMV with *Bean common mosaic potyvirus* (BCMV), *Bean yellow mosaic potyvirus* (BYMV) and *Soybean mosaic potyvirus* (SMV). They also categorised strains of BlCMV in to two distinct species *Bean common mosaic virus* (BCMV) and *Bean common mosaic necrosis virus* (BCMNV). Similiarly, Bhadhramurthy and Bhat (2009) reported that BCMV isolate infecting vanilla showed maximum identity of 96 and 98 per cent at nucleotide and amino acid level, respectively, with Blackeye (Blk1) strain of BCMV indicating its closeness to cowpea infecting BCMV isolates.

5.2. *Papaya ring spot virus* (PRSV)

5.2.1. SYMPTOMATOLOGY

The results of symptomatology revealed that PRSV inoculated plants, of both the resistant and susceptible genotypes developed the initial symptom of water soaked oily vein clearing symptoms (14 DAI), on the younger leaves. However delayed symptom was noticed in the resistant Pusa Nanha. Symptom development on the resistant cultivar, Pusa Nanha was in line with the report made by Chavan *et al*. (2010). They recorded a disease incidence of 44.8 per cent in Pusa Nanha which was very low, compared with the eight commercial papaya cultivars grown in India. Similarly, Sudha *et al.* (2015) recorded a moderate ELISA titre value of 0.962 for Pusa Nanha on mechanical inoculation with PRSV.

Local variety on inoculation with PRSV developed wide range of symptoms including the prominent and characteristic diagnostic symptom of shoe string symptom on leaves and ring spot symptoms on fruits and leaves. Leaves developed mosaic symptoms, became puckered and completely distorted. Fruits of the infected trees became disfigured with bumps on them. There was reduction

in plant canopy, stunted plant growth and finally the death of the plant was observed in case of severe infection. Similar symptoms were described by Gaskill *et al.* (2002); Gonsalves *et al.* (2010); Singh and Shukla (2012). Rahman *et al.* (2008) described the seven distinctly defined symptoms caused by PRSV isolates from Bangladesh, namely, mild mosaic, mosaic, fern leaf, severe mosaic, vein clearing, leaf distortion and chlorotic leaf spot.

5.2.2. TRANSMISSION STUDIES

The present investigation revealed 100 per cent transmission of PRSV on mechanical inoculation. The results were in concurrence with the reports of mechanical transmission of 60 (Bayot *et al.,* 1990), 80 per cent (Kelaniyangoda and Madhubashin, 2010) and 100 (Omar *et al*., 2011). The variations may be due to change in cultivar, virus strain and environmental conditions.

Seed transmission study conducted revealed that PRSV was not seed borne. The observation was in accordance with previous reports (Opina, 1986; Reddy *et al.,* 2007). Contradictory to the present finding, Bayot *et al*. (1990) reported that, PRSV was seed borne to a tune of 0.15 per cent transmission. The reason for variation in seed transmission was governed by many factors such as crop variety, virus strain, temperature and time of infection (Gibbs and Harrison, 1976).

Insect transmission studies revealed that aphids and papaya mealy bug were potential vectors of PRSV. The most efficient one was papaya mealy bug (*Paracoccus marginatus* Williams and Garnara de Willink) (80 per cent) followed by *Aphis gossypii* (40 per cent) and *Aphis craccivora* (30 per cent). Concurrent to the present findings, Kalleshwaraswamy and Kumar (2008) opined that *A. gossypii* (53 per cent) was significantly more efficient in transmitting PRSV than *A. craccivora* (38 per cent). Taya and Singh (1997) reported that, unstarved apterous adults of *A. craccivora* transmitted the PRSV up to 25 per cent.

5.2.3. HOST PATHOGEN INTERACTION STUDIES

5.2.3.1. Estimation of Carbohydrate

The carbohydrate contents were reduced in papaya plants of both genotypes inoculated which were challenge inoculated with PRSV. However carbohydrate content in Pusa Nanha was significantly higher compared to the local variety. The findings related to reduction in total carbohydrates were in concurrence with those reported by earlier workers (Adomako and Hutcheon, 1974; Ashraf and Zafar, 2000; Gonsalves *et al.,* 2005; Handford and Carr, 2007; Goodman *et al*., 2008). Singh and Shukla (2009) documented a significant reduction in reducing sugars, non-reducing sugars and starch content due to PRSV infection.

5.2.3.2. Estimation of Chlorophyll

Total chlorophyll content of both genotypes showed a significant reduction on challenge inoculation with PRSV. Chlorophyll a, Chlorophyll b and total chlorophyll contents of inoculated plants of Pusa Nanha were significantly higher than the inoculated susceptible local variety. Similar, reductions in chlorophyll contents have also been reported in other plant-virus combinations (Funayama-Noguchi and Terashima, 2006; Singh and Shukla, 2009; Arora *et al*., 2009; Sinha and Srivastava, 2010). Muqit *et al.* (2007) observed a reduced chlorophyll content in ash gourd due to infection by *Bottle gourd mosaic virus* (BgMV), *Watermelon mosaic virus*-2 (WMV-2) and PRSV.

5.2.3.3. Estimation of Phenol

Pusa Nanha on inoculation exhibited a significant increase over the uninoculated plants with the maximum value recorded at 10 DAI (10.21 mg g^{-1}). However, it was found to differ non-significantly in inoculated plants of susceptible genotype, with a maximum value recorded at 60 DAI (5.51 mg g^{-1}). The high level of phenolic content in the inoculated plants can be correlated with the increased level of resistance to PRSV, as the accumulation of total phenols

was reported to be higher in resistant genotypes compared to susceptible ones (Pradeep and Jambhale 2002; Ghosal *et al.* 2004; Meena *et al.* 2008; Singh *et al*. 2010).

5.2.3.4. Estimation of Total Soluble Protein

Involvement of protein content in plant diseases resistance have been reported in many host pathogen interactions (Mathur and Shukla, 1976; Wijeendra *et al.*, 1995). Accumulation of protein in Pusa Nanha was found to increase from 5 DAI (1.87 mg g^{-1}) to 60 DAI (7.09 mg g^{-1}), whereas protein accumulation in local variety showed a decreasing trend over the days of inoculation (7.40 at 5 DAI to 3.83 mg g^{-1}at 60 DAI). Concurrent with the findings of present study, Singh and Shukla (2012) have found 10.10 per cent increase in the protein content in the PRSV infected plants, compared to healthy control. Muquit *et al.* (2007) reported that percentage of carbon, nitrogen and protein increased due to PRSV infection. In the present investigation the plants showed a high protein content, which could be due to the activation of both host defense mechanisms and the pathogen attack (Agrios, 1997). Shivaprasad *et al*. (2005); Szczepanski and Redolfi (2008) opined that increased respiration associated with the diseased plants led to the synthesis of more amino acids, leading to protein accumulation.

5.2.3.5. Estimation of Defense Related Enzymes

Defense related enzymes were reported to play an important role in the induction of disease resistance (Dasgupta, 1988). PO was one of the first enzymes to get activated in the defense against plant pathogens (Ye *et al*., 1990). The peroxidase activity of Pusa Nanha reached the maximum at 10 DAI (20.42 min^{-1} g^{-1}) and slowly declined over the days. However, susceptible genotype recorded the maximum activity at 5 DAI (25.90 min^{-1} g^{-1}) and slowly declined over the days. Similar increase in PO activity in resistant genotypes, was observed in CMV and *Zucchini yellow mosaic virus* (ZYMV) infected *Cucumis sativus* and *Cucurbita pepo* plants (Riedle-Bauer, 2000). In diseased samples the peroxidase activity was higher in the early stage of symptom expression due to

oxidation of phenolic compounds and other metabolites. Enzyme activity declined at later stage of infection due to destruction of cell organelles, which was associated with deficiency of metabolites as opined by Shilpasree *et al*. (2012).

The results revealed that PPO activity in resistant cultivar was similar to that of PO over the days of inoculation. However the enzyme activity showed a non-significant difference in susceptible local variety (Fig 16b). Induction of PO and PPO was in line with observation of Chang and Yan (2006) who reported higher activity of enzymes in the natural resistant mutant of PRSV, compared to susceptible cultivar, Suizhonghong. Hui-hua *et al.* (2007) have also reported higher activity of these enzymes in resistant papaya genotype, Malai-10 than the susceptible genotype, Tainong-2.

The results revealed that, PAL activity was significantly higher in the inoculated plants of Pusa Nanha (98.02 µg $g^{-1}min^{-1}$ at 5 DAI to 75.44 µg g^{-1} min^{-1} at 60 DAI), compared to the inoculated susceptible genotype. On the other hand the activity was found to decrease significantly (76.64 µg g^{-1} min^{-1} at 5 DAI to 49.20 µg g^{-1} min^{-1} at 60 DAI) in the inoculated plants of susceptible genotype. Similar increase in PAL activity in resistant cultivar, was observed in cotton infected with *Cotton leaf curl virus* (CLCuV) (Siddique *et al.*, 2014). A possible explanation for decrease in enzyme activity after PRSV inoculation in susceptible genotype is that, many plant pathogens actively suppress the expression of plant defense reactions during successful infection (Markakis *et al.*, 2010). Results revealed a positive correlation between increased enzyme activity (PO, PPO and PAL) and phenol accumulation in resistant genotype, upon PRSV inoculation. Hence, it can be concluded that the three key enzymes along with phenol play a crucial role in disease resistance against PRSV.

5.2.3.6. Electrophoretic Analysis of Proteins using SDS-PAGE

Nine induced proteins (98, 34, 30, 27, 25, 18, 15, 12 and 10 kDa) were found in resistant Pusa Nanha. Eight proteins (34, 10, 30, 29, 27, 25, 18 and 15 kDa) were induced in the susceptible local variety of papaya. The proteins

detected in the study having molecular weights in the range 32 to 36 kDa are similar in size to those detected previously (Gonsalves and Ishi, 1980; Kunkalikar *et al*., 2006; Tripathi *et al.,* 2008). The extra band with molecular weight of 34 kDa was suggested to be the capsid protein of PRSV. The observation was in accordance with the observation of Roy *et al.* (1999) who identified that virus particles in PRSV migrated as a single polypeptide of apparent molecular weight 34 kDa. Nambisan (1996) opined that newer proteins identified in response to virus inoculation were some low molecular weight stress induced proteins.

Electrophoretic patterns of soluble proteins have been used as a powerful tool for the study of genetic variability of *Solanum* species. The profiles of tuber soluble proteins were applied in the studies of plant genetics of cultivated and wild potato species and hybrids (Giovannini *et al*., 1993; Kormu *et al*., 1999). Adi *et al.* (2012) compared protein profiles and metabolites patterns in *Tomato yellow leaf curl virus* (TYLCV) infected resistant and susceptible tomatoes, which revealed a completely different host stress response. Susceptible plants were characterized by higher levels of reactive oxygen species (ROS) and ROS compounds, anti-oxidative, pathogenesis-related (PR) and wound-induced proteins. In contrast, infection on resistant tomatoes did not drastically activated these host defense mechanisms. However, the homeostasis was effectively maintained by protein and chemical chaperones.

5.2.3.7. Electrophoretic Analysis of Isozyme

Isoperoxidases with Rm values of 0.08 and of 0.23 were unique for the resistant genotype, Pusa Nanha. However no unique isozyme was identified for susceptible local variety. Low activity of isozyme ‘a’ for local variety upon inoculation with PRSV, suggested on the inactivation of initial defense responses. Similarly, Rathi *et al*. (1986) identified specific isoperoxidases for resistant (Hy3C) and susceptible (Type-21) pigeon pea genotypes systemically infected with *Pigeon pea sterility mosaic virus* (PPSMV). Altered zymogram pattern of isoperoxidases of susceptible variety, following infection, suggested inactivation

of existing isoperoxidases, activation of inactive form and/or synthesis of new isoperoxidases (Shaw *et al*., 1994; Valencia *et al*., 2001).

5.2.4. BIOLOGICAL DETECTION

Chenopodium quinoa on sap inoculation produced numerous local chlorotic lesions and was identified as an indicator plant for the biological detection of PRSV. However *Chenopodium amaranticolor* failed to develop any local lesions. The results obtained were in agreement with previous reports (Bayot *et al*., 1990; Natsuaki, 2011). Kelaniyangoda and Madhubashin (2010) reported that, out of ten plant species observed after inoculation with PRSV, *C. quinoa* was the only potential indicator plant, with 50 per cent transmission. Contradictory to the present findings, Perera *et al*. (1998); Roy *et al.* (1999) identified *C. amaranticolor* as an indicator plant for PRSV.

5.2.5. IMMUNOLOGICAL STUDIES

DAC-ELISA was successfully used to detect PRSV isolate from Kerala, using polyclonal antibody against PRSV and *Potyvirus* group antiserum. The absorbance values (PRSV) from the study revealed that mosaic symptom (1.307) caused by PRSV had the highest viral inoculum followed by shoestring (1.241) and ring spot symptom (1.183). In concurrence with the present study, Roy *et al.* (1999) Singh and Shukla (2012) identified PRSV isolates using DAC-ELISA.

DIBA using polyclonal antibody against PRSV, also detected PRSV isolate from Kerala. Similarly, DIBA was used for the detection of PRSV infection in cucurbitaceous crops (Maura *et al.*, 2001) sesamum (Singh *et al*., 2006) and papaya (Singh and Shukla, 2012).

5.2.6. MOLECULAR DETECTION AND CHARACTERISATION

The PCR product of 220 bp size was sequenced and analysed using Basic Local Alignment Search Tool (BLAST) software (Fig.10 and Table 36). Comparative nucleotide sequence alignment of the virus with the data bases available from NCBI revealed a 99 per cent homology with PRSV isolate from Andhra Pradesh. The isolate also shared 93 per cent homology with *Papaya ring*

spot virus isolate biotype W from Brazil. Comparative amino acid sequence alignment revealed a 100 per cent homology with coat protein of PRSV. Comparative sequence analysis of BlCMV and PRSV *Potyviruses* showed that the sequences were entirely different and showed only a 51.12 per cent similarity at nucleotide levels.

Similar to the present observations, Jain *et al.* (2004) identified considerable heterogeneity in the *Papaya ring spot virus* (PRSV) isolates CP sequences and CP-coding region varying in size from 840 to 858 nucleotides, encoding protein of 280 to 286 amino acids. Bag *et al.* (2007) identified PRSV isolates from central, eastern, northern, southern and western India as highly heterogeneous in CP length (275 to 289 amino acids) and amino acid sequences (up to 23 per cent). Srinivasulu and Saigopal (2011) reported a sequence divergence of 18.4 and 15 per cent at nucleotide and amino acid levels, respectively within the Indian PRSV populations.

Summary

6. SUMMARY

The *Potyviruses* causing mosaic in cowpea and ring spot in papaya were selected for the study owing to their widespread occurrence among the cultivars in Kerala. The present study entitled "Immunomolecular detection and characterisation of *Potyviruses* infecting cowpea (*Vigna unguiculata* (L.) Walp.) and papaya (*Carica papaya* L.)" was undertaken in the Department of Plant Pathology, College of Agriculture, Vellayani during 2013-2015 with an objective to study the symptomatology and transmission of *Potyviruses* infecting cowpea and papaya, along with their biological, immunological, molecular detection and characterisation.

Symptoms caused by *Blackeye cowpea mosaic virus* (BlCMV) in cowpea included mosaic and vein banding, followed by distortion and reduction in leaf size. The plants also showed severe stunting and resulted in reduction in number of flowers and fruits.

Symptoms caused by *Papaya ring spot virus* (PRSV) in papaya included vein clearing, mosaic, puckering and shoe string on leaves. Infected leaf became completely disfigured, with marked size reduction. Complete rings or broken rings and chlorotic spots (ring spots) were also observed on leaves and fruits.

The symptoms produced on cowpea and papaya plants by mechanical inoculation resembled those observed naturally under field condition. The cowpea and papaya plants mechanically inoculated with BlCMV and PRSV developed the initial symptom of vein clearing, on the younger leaves 7 and 14 days after inoculation (DAI) respectively.

BlCMV and PRSV were mechanically transmissible (80 and 100 per cent respectively). Seed transmission (48.5 per cent) was recorded for BlCMV in cowpea, whereas PRSV was not seed borne. BlCMV was transmitted by *Aphis craccivora* Koch and *Aphis gossypii* Glover (30 per cent respectively). Papaya mealy bug (*Paracoccus marginatus* Williams and Garnara de Willink) (80 per

cent) was the most efficient vector of PRSV followed by *A. gossypii* (40 per cent) and *A. craccivora* (30 per cent).

Host pathogen interaction studies in resistant and susceptible genotypes of cowpea (CO6 and Vellayani Jyothika) and papaya (Pusa Nanha and local variety) indicated a significant reduction in total carbohydrates and chlorophyll contents in susceptible genotypes on inoculation with *Potyviruses*, whereas resistant genotypes showed no significant difference. However, phenol, protein and defense related enzymes showed a significant increase in resistant genotypes on inoculation, compared to the susceptible genotypes.

Delayed symptom expression followed by remission in resistant cultivars (CO6 and Pusa Nanha) were noticed for the *Potyviruses*.

Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) and native-PAGE were conducted for identifying induced proteins and isozymes respectively, in resistant and susceptible genotypes of cowpea and papaya. Eleven novel proteins (98, 96, 32, 30, 27, 19, 15, 14, 13, 11 and 8 kDa) were induced in resistant CO6. Ten new proteins (97, 43, 32, 22, 20, 18, 14, 11, 10 and 8 kDa) were observed in susceptible Vellayani Jyothika.

Nine induced proteins (98, 34, 30, 27, 25, 18, 15, 12 and 9 kDa) were found in resistant Pusa Nanha. Eight proteins (34, 30, 29, 27, 24, 16, 14 and 10 kDa) were induced in the susceptible local variety of papaya.

Isoperoxidases with Relative mobility (Rm) values of 0.13, 0.20 and 0.27 were unique for CO6. There was no unique isozyme on inoculation in Vellayani Jyothika.

Isoperoxidases with Rm values of 0.08 and 0.23 were unique for Pusa Nanha. However no specific isozyme was observed in local variety of papaya.

Chenopodium amaranticolor and *Chenopodium quinoa* were identified as indicator plants for the biological detection of BlCMV and PRSV.

Immunological and molecular characterisation for the *Potyviruses* infecting cowpea and papaya were done. Direct antigen coating-Enzyme linked immunosorbent assay (DAC-ELISA) and Dot immunobinding assay (DIBA) efficiently detected the viruses. Polyclonal (H2) and two monoclonal antibodies specific to BlCMV (15E6 and 16G5) gave a positive reaction in symptomatic plants using DAC-ELISA. The polyclonal and monoclonal antibodies specific to CABMV gave a negative reaction indicating the virus causing mosaic in cowpea was BlCMV. DAC-ELISA using polyclonal antibody against PRSV and *Potyvirus* group antiserum, detected PRSV isolate. The vector role of papaya mealy bug was confirmed using DAC-ELISA.

DIBA using polyclonal antibodies against BlCMV (H2) and PRSV also detected virus isolates.

Molecular detection *viz.* Reverse transcription-Polymerase chain reaction (RT-PCR) using BlCMV and PRSV specific primers produced an amplicon of sizes ~110 and ~220 bp respectively, identifying the *Potyviruses* as BlCMV and PRSV respectively.

Comparative nucleotide sequence alignment of the viruses with the available data bases from National Centre for Biotechnology Information (NCBI) revealed a 97 per cent homology with *Bean common mosaic virus* (BCMV) strain Blackeye from Karnataka and 99 per cent homology with PRSV isolate from Andhra Pradesh respectively. Comparative amino acid sequence alignment revealed a 95 and 100 per cent homology with coat proteins of BCMV and PRSV respectively.

The present investigation revealed both viruses were mechanically transmitted and by insect vectors associated with the crops. Present study also revealed that BlCMV was transmitted through seeds, while PRSV was not seed transmitted. Host pathogen interaction studies indicated that phenol, protein, defense related enzymes, could be exploited as biochemical markers to study plant virus interactions. Induced proteins and activated isoforms of peroxidase

specifically identified in resistant and susceptible cultivars could be used as markers to identify resistance and susceptibility. The present molecular study confirmed that BlCMV infecting cowpea was closely related to BCMV, a strain of Blackeye. PRSV in the present investigation was found related to PRSV isolates reported from other parts of India. Comparative sequence analysis of BlCMV and PRSV *Potyviruses* showed that the sequences were entirely different and showed only a 51.12 per cent similarity at nucleotide levels. Serological and sequence studies thus suggest that BlCMV caused the mosaic disease in cowpea and PRSV, the ring spot disease in papaya.

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APPENDIX-1

BUFFERS FOR SAP EXTRACTION

1. 0.1 *M* Sodium phosphate buffer (pH 7.0)

Stock solutions

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

B: 0.2 *M* Solution of dibasic sodium phosphate (53.65 g of $Na_2.HPO_4.12\ H_2O$ in 1000 ml)

39 ml of A is mixed with 61.0 ml of B diluted to a total of 200 ml.

APPENDIX-11

ESTIMATION OF CARBOHYDRATE

1. Anthrone reagent

Anthrone reagent made by dissolving 200 mg of anthrone in 100 ml of ice cold 95% concentrated Sulphuric acid.

APPENDIX-111

ESTIMATION OF PROTEIN

1. 0.1 *M* Sodium acetate buffer (pH 4.7)

Stock solutions

A: 0.2 *M* solution of Acetic acid (11.55 ml in 1000 ml)

B: 0.2 M solution of Sodium acetate (16.4 g of $C_2H_3O_2$ Na or 27.2 g of $C_2H_3O_2Na.3H_20$ in 1000 ml)

22.7 ml of A is mixed with 27 ml of B, diluted to a total of 100 ml.

2. Preparation of stock dye solution for estimation of protein

100 mg of Coomassie brilliant blue G-250 was dissolved in 50 ml of 95 % ethanol and 100 ml of concentrated Orthophosphoric acid was added. The volume was made up to 200 ml with water and kept at 4 ^oC. The working dye was prepared just before use by diluting the stock solution to five times with water.

APPENDIX IV

BUFFERS FOR ENZYME ANALYSIS

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

Stock solutions

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

B: 0.2 *M* Solution of dibasic sodium phosphate (53.65 g of $Na_2.HPO_4.12\ H_2O$ in 1000 ml)

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

2. 0.1 *M* Borate buffer (pH 8.8)

Stock solutions

A: 0.2 *M* solution Boric acid (12.4 g in 1000 ml)

B: 0.05 *M* 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 V

ELECTROPHORETIC ANALYSIS OF PROTEINS USING SDS-PAGE

1. Protein denaturing solution

10 *M* urea	-80 ml
1 *M* $NaH_2PO_4.2\ H_2O$ (pH 8)	-5 ml
1*M* Tris (pH 8)	-1 ml
5 *M* Sodium chloride	-2 ml

Make up the volume to 100 ml by adding 12 ml of distilled water.

2. Acrylamide stock (30 %)

Acrylamide	-29.2 g
Bis-acrylamide	-0.8 g
Double distilled water	-100.0 ml

3. Separating (resolving) gel buffer stock (1.5 *M* Tris-HCl, pH 8.8)

Tris-base (18.15 g) was dissolved in approximately 50 ml of double distilled water. The pH was adjusted to 8.8 with 6 *N* HCl and volume was made up to 100 ml with double distilled water and stored at 4 °C.

4. Stacking gel buffer stock (0.5 *M* Tris-HCl pH 6.8)

Tris base (6.0 g) was dissolved in approximately 60 ml of double distilled water. The pH was adjusted to 6.8 with 6 *N* HCl and the volume was made up to 100 ml with double distilled water and stored at 4°C.

5. Polymerising agents

Ammonium per sulphate (APS) - 10 % prepared freshly before use.

TEMED - fresh from refrigerator

6. Electrode buffer (pH 8.3)

Tris base	-6.0 g
Glycine	-28.8 g
SDS	-2.0 g
Double distilled water	-2 L

7. Sample buffer

Double distilled water	-2.6 ml
0.5 M Tris-HCl (pH 6.8)	-1.0 ml
2-Mercaptoethanol	-0.8 ml
Glycerol	-1.6 ml
SDS 20 per cent (w/v)	-1.6 ml
0.5 % Bromophenol blue	-0.4 ml

8. Staining solution

Coomassie brilliant blue R 250	-0.1 g
Methanol	-40.0 ml
Glacial acetic acid	-10.0 ml
Double distilled water	-50.0 ml

9. Destaining solution

As above without Coomassie brilliant blue R 250.

10. Preparation of separating gel (12 %)

Double distilled water	-6.7 ml
Tris HCl (pH 8.8)	-5.0 ml
SDS 10 per cent	-0.2 ml
Acrylamide stock	-8.0 ml

The above solution was mixed well and degassed for 3 min and then the following were added immediately.

10 % freshly prepared Ammonium per sulphate (APS) -0.10 ml

Tetra methyl ethylenediamine (TEMED) -0.01 ml

The separating gel was mixed well and poured immediately between glass plates and a layer of water was added above the polymerizing solution to quicken the polymerization process.

11. Preparation of stacking gel (4 %)

Double distilled water -6.1 ml

Tris HCl (pH 6.8) -2.5 ml

SDS 10 per cent -0.2 ml

Acrylamide stock -1.3 ml

The solution was mixed well, degassed and the following were added.

APS 10 % -0.05 ml

TEMED -0.1 ml

APPENDIX VI

ELECTROPHORETIC ANALYSIS OF ISOZYME

1. **Tris-glycine electrode buffer stock solution (pH 8.3)**

   Tris -6.0 g

   Glycine -28.8 g

   Distilled water -1000 ml

2. **Electrode buffer**

   Dilute the Tris-glycine electrode buffer stock solution with distilled water in a ratio of 1:9 ratio.

3. **Tris-chloride buffer stock solution (pH 8.9)**

   HCl, 1 *N* -48.00 ml

   Tris -36.6 g

   TEMED -0.23ml

   Distilled water -100.00 ml

4. **Tris-chloride buffer stock solution (pH 6.7)**

   HCl, 1 *N* -48.00 ml

   Tris -5.98 g

   TEMED -0.46 ml

   Distilled water -100.00 ml

5. **Resolving gel acrylamide stock solution**

   Acrylamide -28.00 g

   Bis-acrylamide -0.74 g

   Double distilled water -100.00 ml

   Store in dark bottle at 4 ^{0}C for up to 2 weeks.

6. **Ammonium persulphate solution**

   Ammonium persulphate -0.1 g

   Dissolve in 1 ml distilled water. Prepared freshly before use.

7. Bromophenol blue solution

Bromophenol -25 mg

Make up to 10 ml with Tris-chloride buffer solution (pH 6.7).

8. Resolving gel solution (for one 1.5 mm gel, 7.5%)

Tris-chloride buffer stock (pH 8.9) -5 ml

Resolving gel acrylamide solution -10 ml

Triton X-100 -2 %

Distilled water -25 ml

Ammonium persulphate solution -300µl

9. Stacking gel solution (for one 1.5 mm gel, 4%)

Tris-chloride buffer stock (pH 6.7) -2.5 ml

Resolving gel acrylamide solution -10 ml

Triton X-100 -2 %

Distilled water -25 ml

Ammonium persulphate solution -300µl

10. Separating gel (7.5 %)

Tris chloride buffer stock (pH 8.9) -5 ml

Resolving gel acrylamide solution -10 ml

Distilled water -25 ml

APS -300 µl

11. Stacking gel (4 %)

Tris chloride buffer stock (pH 6.7)	-2.5 ml
Resolving gel acrylamide solution	-3.1 ml
Distilled water	-14.1 ml
APS	-300 μl

APPENDIX VII

BUFFERS FOR DAC-ELISA

1. Phosphate buffered saline (PBS-pH 7.4)

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

2. Wash Buffer

Add 0.5 ml/L of Tween-20 to PBS

3. Coating Buffer (pH 9.6)

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

4. Antibody diluent buffer

Add 20 g PVP and 2 g ovalbumin to 1 L PBS-T

5. Enzyme conjugate diluent buffer (PBS-TPO)

Same as PBS-TPO.

6. Substrate solution (pH 9.8)

Diethanolamine	-97 ml
Sodium azide	-0.2 g
Water	-800 ml

Add HCl to attain the required pH (9.8).

APPENDIX VIII

STOCK SOLUTIONS FOR DIBA

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

0.02 M Tris	-4.84 g
0.5 M NaCl	-58.48 g

Adjust the pH to 7.5 with 1 N HCl and make up to 2 litre. This is used as wash solution.

2. Antigen extraction buffer (TBS- 500 m*M* DIECA)

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

3. Blocking solution (TBS-SDM)

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

4. Antibody and enzyme –conjugate diluent/buffer

Same as TBS-SDM.

5. Substrate buffer (pH 9.5)

0.1 *M* Tris	-12.11 g
0.1 *M* NaCl	-5.85 g
5 m*M* $MgCl_2$. $6H_20$	-1.01 g

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

6. Substrate solution

Solution A

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

Solution B

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

Store solutions A and B refrigerated in amber coloured bottles. Add 44µl of NBT and 35µl of BCIP to 10 ml substrate buffer.

7. Fixing solution (pH 7.5)

10 m*M* Tris	-1.21 g
1 m*M* EDTA	-0.29 g

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

APPENDIX IX

BUFFERS FOR PCR ANALYSIS

1. 50 x TAE buffer (Tris –Acetate-EDTA) (pH 8.0)

Tris base	-242.0 g
Acetic acid	-57.1 ml
0.5 *M* EDTA	-100 ml

Add distilled water to a final volume of 1 litre.

2. Sample loading buffer (6x)

0.25% Bromophenol blue

40% (w/v) sucrose in water

APPENDIX X

Gene sequence of the BlCMV isolate under study

ATCAGATGATGCAGCAGCCCTCAGCAACGTTAGCAGCAAGTTGTTTGA
CTTGACGGTAATGTTGCAACAACTCAGCGAGAATACTGAAAGGCACAT
GCAAGGGACGTTAACCAAAAAACGGTATAATTGTT

Gene sequence of the PRSV isolate under study

TTCCTCTGTAAGACTACTACCTGATAGAGCTAGAGAGGCTCATATGCG
ATGAAAGCAGCTGCACTGCGAAACGCAAATCGCAGAATGTTTGGAAG
GACGGCAGTGTCAGTAACAAGGAAGAAAACACGGAGAGACACACAGT
GGAAGATGTCAATAGGGACATGCACTCTCTCCTGGGTA

Abstract

IMMUNOMOLECULAR DETECTION AND CHARACTERISATION OF *POTYVIRUSES* INFECTING COWPEA (*Vigna unguiculata* (L.) Walp.) AND PAPAYA (*Carica papaya* L.)

by

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ABSTRACT

The study entitled "Immunomolecular detection and characterisation of *Potyviruses* infecting cowpea (*Vigna unguiculata* (L.) Walp) and papaya (*Carica papaya* L.)" was conducted at Department of Plant Pathology, College of Agriculture, Vellayani, Thiruvananthapuram during 2013-2015 with the objective to study the symptomatology and transmission of *Potyviruses* infecting cowpea and papaya, along with their biological, immunological, molecular detection and characterisation.

Symptoms caused by *Blackeye cowpea mosaic virus* (BlCMV) in cowpea include mosaic and vein banding and that of *Papaya ring spot virus* (PRSV) in papaya showed mosaic and shoe string on leaves and ring spot on fruits and leaves.

BlCMV and PRSV were mechanically transmitted (80 and 100 per cent respectively). Seed transmission (48.5 per cent) was recorded for BlCMV in cowpea. PRSV was not transmitted through seeds. BlCMV was transmitted by *Aphis craccivora* Koch and *Aphis gossypii* Glover (30 per cent respectively). Papaya mealy bug (*Paracoccus marginatus* Williams and Garnara de Willink) (80 per cent) was the most efficient vector of PRSV followed by *Aphis gosssypii* Glover (40 per cent) and *Aphis craccivora* Koch (30 per cent).

Host pathogen interaction studies in resistant and susceptible genotypes of the cowpea (CO6 and Vellayani Jyothika) and papaya (Pusa Nanha and local variety) indicated a significant reduction in total carbohydrates and chlorophyll contents in susceptible genotypes on inoculation with *Potyviruses*, whereas resistant genotypes showed no significant difference. However, phenol, protein and defense related enzymes showed a significant increase in resistant genotypes on inoculation, compared to the susceptible genotypes.

Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) and native-PAGE were conducted for the comparison of proteins and

isozymes respectively, in resistant and susceptible genotypes. Eleven novel proteins were induced in CO6 whereas ten new proteins were observed in Vellayani Jyothika. Nine and eight induced proteins were identified in Pusa Nanha and local variety of papaya respectively.

Three isoperoxidases with Relative mobility (Rm) values of 0.13, 0.20 and 0.27 were unique for CO6. There was no unique isozyme on inoculation in Vellayani Jyothika. Isoperoxidases with Rm values of 0.08 and 0.23 were unique for Pusa Nanha. However no specific isozyme was observed in local variety of papaya.

Chenopodium amaranticolor and *Chenopodium quinoa* were identified as indicator plants for the biological detection of BlCMV and PRSV. Immunological studies using Direct antigen coating-Enzyme linked immunosorbent assay (DAC-ELISA) and Dot immunobinding assay (DIBA) and molecular detection *viz.* Reverse transcription-Polymerase chain reaction (RT-PCR) identified the *Potyviruses* as Bean common mosaic virus (BCMV) strain Blackeye and PRSV respectively. Comparative amino acid sequence alignment revealed a 95 and 100 per cent homology with coat proteins of BCMV and PRSV respectively.

The transmission studies revealed that *Potyviruses* were transmitted mechanically and by insects. Present study also revealed that BlCMV was transmitted through seeds while, PRSV was not seed transmitted. The host pathogen interaction studies identified phenol, protein and defense related enzymes along with induced proteins and isoforms of peroxidase could be used as biochemical markers to identify resistance and susceptibility in plants. The present molecular study confirmed that BlCMV infecting cowpea was closely related to BCMV, a strain of Blackeye. PRSV in the present investigation was found related to PRSV isolates reported from other parts of India. Comparative sequence analysis of BlCMV and PRSV *Potyviruses* showed that the sequences were entirely different and showed only a 51.12 per cent similarity at nucleotide

levels. Serological and sequence studies thus suggest that BlCMV caused the mosaic disease in cowpea and PRSV, the ring spot disease in papaya.