Etiology and management of mosaic disease in ginger
(*Zingiber officinale* Roscoe)

by

ANANTHU. N

(2015 - 11 - 094)

THESIS

Submitted in partial fulfillment of the

requirements for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF PLANT PATHOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM-695522

KERALA, INDIA

2018

DECLARATION

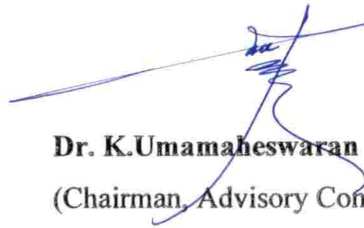
I, hereby declare that this thesis entitled **"Etiology and management of mosaic disease in ginger (Zingiber officinale Roscoe)"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Vellayani, **Ananthu. N**

Date: 20.06.2018 **(2015 -11-094)**

CERTIFICATE

Certified that this thesis **entitled "Etiology and management of mosaic disease in ginger (Zingiber officinale Roscoe)"** is a record of research work done independently by Mr. Ananthu. N. under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.



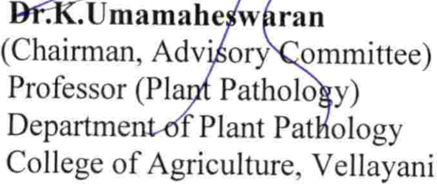
Place: Vellayani

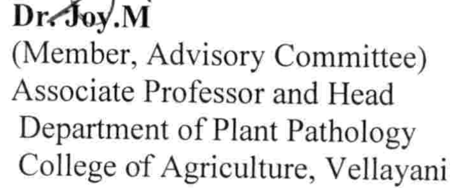
Date: 20.6.18

Dr. K.Umamaheswaran
(Chairman, Advisory Committee)
Professor (Pl. Path.)
Department of Plant Pathology
College of Agriculture, Vellayani.

CERTIFICATE

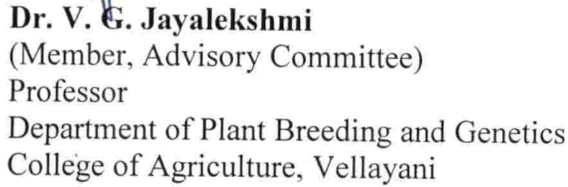
We, the undersigned members of the advisory committee of Mr. Ananthu. N., a candidate for the degree of **Master of Science in Agriculture** with major in Plant Pathology, agree that the thesis entitled **"ETIOLOGY AND MANAGEMENT OF MOSAIC DISEASE IN GINGER (ZINGIBER OFFICINALE ROSC.)"** may be submitted by Mr. Ananthu. N., in partial fulfillment of the requirement for the degree.

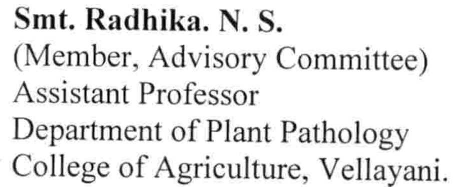




Dr.K.Umamaheswaran
(Chairman, Advisory Committee)
Professor (Plant Pathology)
Department of Plant Pathology
College of Agriculture, Vellayani

Dr. Joy.M
(Member, Advisory Committee)
Associate Professor and Head
Department of Plant Pathology
College of Agriculture, Vellayani





Dr. V. G. Jayalekshmi
(Member, Advisory Committee)
Professor
Department of Plant Breeding and Genetics
College of Agriculture, Vellayani

Smt. Radhika. N. S.
(Member, Advisory Committee)
Assistant Professor
Department of Plant Pathology
College of Agriculture, Vellayani.

EXTERNAL EXAMINER

डॉ. टी. मकेशकुमार, पीएच.डी.
Dr. T. MAKESHKUMAR, Ph.D.
प्रधान वैज्ञानिक
Principal Scientist (Plant Patholo

ACKNOWLEDGEMENT

First of all I express my heartfelt gratitude and indebtedness to the supreme power for his blessings to overcome each and every obstacles faced and finally to triumph in my present endeavour.

I am pleased to place my esteem and deep sense of gratitude towards Dr. K. Umamaheswaran, Professor, Department of Plant Pathology and honoured chairman of my advisory committee for his valuable guidance, scholarly suggestions, unfailing patience, moral support and help rendered wholeheartedly by him throughout the period of work.

I am immensely grateful to Dr. M. Joy, Associate Professor and Head, Department of Plant Pathology for his support with valuable guidance, advice and encouragement.

I accord my sincere thanks to my advisory committee members Dr. V. G. Jayalekshmi, Professor, Department of Plant Breeding and Genetics and Smt. Radhika. N. S., Assistant Professor, Department of Plant Pathology for their guidance and valuable suggestions extended during the work.

It is with immense gratitude, I acknowledge Dr. C. Gokulapalan, Dr. V. K. Girija, Dr. Lulu Das, Former Heads of the department for their wholehearted support and guidance.

I also accord my sincere thanks to Dr. Susha. S. Thara and Dr. Heera. G., my beloved and respected teachers of Plant Pathology for their immense help and encouragement.

My heartfelt thanks to all the non-teaching staff of Department of Plant Pathology for their cooperation.

My sincere thanks to Dr. A. Ishwara Bhat, Scientist Indian Institute of Spices Research, Kozhikode for his valuable suggestions for the research work.

I feel dearth of words to express my gratitude to my dear friends Aparna, Chinnu, Brinda, Karthika, Hari, Jaslam, Dhanesh, Rejeth, Vishnu, Narayanan, Amogh and Manjunath for their wholehearted help and cooperation during my research work.

Words fail to acknowledge Smitha Chechi for her selfless help and support rendered to me during the research work, and I also acknowledge my dear seniors Asha Chechi, Manal Chechi, Safeer Chettan, Krishnapriya Chechi, Theresa Chechi, Parvathy Chechi, Vijeth bhai and Gireesh Chettan for their support and cooperation.

I wish to pledge my sincere thanks to my dear juniors Naveen, Agnes, Anjana, Geethu, Madhu, Sooraj, Rahul, Shilpa, Bhavana, Deepa, Bincy, Deepthi, Safana, Athira, Jyothi, Chandran and Pavan for their cooperation and support.

Finally I wish to register my deepest and utmost gratitude to my dearest Achan, Amma, Vishnu, Latha Chitta, Aphan, Unniyettan, Vidya Edathi, Ammu and Arjun for standing beside me throughout all the tumultuous times I have been through and for all the immense emotional strength they gave me to face all my fears. I am truly indebted to them for all that I am today.

Ananthu. N

CONTENTS

Sl. No.	CHAPTER	Page No.
1	INTRODUCTION	1
2	REVIEW OF LITERATURE	3
3	MATERIALS AND METHODS	21
4	RESULTS	42
5	DISCUSSION	59
6	SUMMARY	73
7	REFERENCES	76
	ABSTRACT	
	APPENDICES	

LIST OF TABLES

Table No.	Title	Page No.
1	Changes in total carbohydrate content of ginger leaves due to viral infection	44
2	Changes in total chlorophyll content of ginger leaves in response to virus infection	45
3	Changes in phenol content of ginger leaves in response to virus infection	46
4	Changes in total soluble protein content of ginger leaves in response to virus infection	47
5	Changes in peroxidise activity in ginger leaves in response to virus infection	48
6	Changes in polyphenol oxidase activity in ginger leaves in response to virus infection	49
7	Changes in phenylalanine ammonialyase activity in ginger leaves in response to virus infection	50
8	Reaction of ginger samples infected by virus in DAC-ELISA	51
9	Reaction of ginger samples infected by virus in TAS- ELISA	52
10	Description of selected isolates for the construction of cluster dendrogram	56
11	Vulnerability index (V.I.) of plants after the management study	58

LIST OF PLATES

Fig. No.	Title	Pages Between
1	Scoring of disease according to symptoms	40-41
2	Appearance of chlorotic flecks in the leaves	42-43
3	Slender spindle shaped streaks	42-43
4	Mosaic pattern on the leaves	42-43
5	Necrotic spots formation	42-43
6	Appearance of symptoms in rhizome transmission	42-43
7	Protein profile analysis using SDS PAGE	50-51
8	Peroxidase isozyme profile	50-51
9	Reaction in DIBA	52-53
10	RNA isolated	52-53
11	DNA isolated	53-54
12	PCR amplification for begomoviruses	53-54
13	BLAST results obtained for the sequence	53-54
14	Cluster dendrogram showing the relationship of begomovirus isolate with related sequences	53-54

LIST OF APPENDICES

Sl. No.	Title	Appendix No.
1	Buffers for sap extraction	I
2	Buffer for protein estimation	II
3	Buffers for enzyme analysis	III
4	Buffer for DNA isolation	IV
5	Buffers for SDS PAGE	V
6	Buffers for isozyme analysis	VI
7	Buffers for PCR analysis	VII
8	Buffers for DIBA	VIII
9	Buffers for ELISA	IX
10	Buffer for molecular gel loading	X
11	PCR Product sequence	XI

LIST OF ABBREVIATIONS AND SYMBOLS USED

%	Per cent
L	Litre
°C	Degree Celsius
CD	Critical Difference
et al.	And other co workers
g	Gram
h	Hours
ml	Milli litre
mM	Milli molar
min.	minutes
mg	Milli gram
Sl. No.	Serial number
spp.	Species
viz.	Namely
pH	Negative logarithm of hydrogen ions
μm	Micro metre
ppm	Parts per million
bp	Base pair
kDa	kilo dalton
kb	kilo base
N	Normal
M	Molar
μL	Micro litre
nm	Nano metre
RT-PCR	Reverse transcription polymerase chain reaction
CRD	Completely randomized design
RNA	Ribonucleic acid
ELISA	Enzyme linked immunosorbant assay
DIBA	Dot immune binding assay
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel melectrophoresis
PBS-TPO	Phosphate buffer saline tween polyvinyl pyrrolidone ovalbumin
DAI	Days after inoculation
rpm	Revolutions per minute
SDM	Spray dried milk
TBS	Tris buffer saline
PO	Peroxidase
PPO	Polyphenol oxidase
PAL	Phenylalanine ammonialyase
NCM	Nitrocellulose membrane
BSA	Bovine serum albumin
TEMED	Tetramethyl ethylene diamine

Introduction

1. INTRODUCTION

Ginger is one of the earliest recognized oriental spices. From time immemorial, it is being cultivated in India for both fresh vegetable and dried spice. Ginger is obtained from the rhizomes of *Zingiber officinale* Rosc. The ginger family is a tropical group, especially abundant in the Indo- Malaysian region. This family consists of more than 1200 plant species in 53 genera. It is one of the oldest spice crops of the world like cinnamon, clove and pepper. It is being cultivated in several parts of the world, mainly in the countries like India, China, Japan, Taiwan, Nigeria, Australia etc.

In India, ginger cultivation covers an area of 281 thousand ha, from which a total of 1109 thousand tons of ginger is produced (National Horticulture Board, 2016). India is the largest producer and exporter of ginger in the world, followed by China and Taiwan (FAO, 2016). Kerala has a good contribution in the total production of ginger. A high proportion of the dry ginger from the state, both bleached and unbleached is exported to overseas countries. India exports ginger mostly to the countries viz. USA, UK, Saudi Arabia, Canada and Singapore. Ginger is used principally as an ingradient in various spice blends, food processing and beverage industries for flavouring coffee and tea.

Ginger requires a warm and humid climate, and soil of high organic matter content, high fertility, proper drainage, good aeration and a pH of 6.0 to 7.0. if all the factors are favourable, it is a highly promising crop and huge loss if unfavourable condition prevails.

A number of diseases are known to cause damage to ginger in India. The important of them are leaf spot, soft rot and bacterial wilt. Among the spice crops the viral diseases are of very little knowledge. The viral diseases are nowadays increasing in spice crops because of their increase in area of cultivation.

In recent years, ginger has been observed to suffer from a mosaic disease throughout the cultivating tracts and also in the fields of Instructional Farm, College of Agriculture, Vellayani. To achieve an integrated and ecofriendly

system for virus disease management, proper detection and identification of the virus has to be done. In view of the importance of viral diseases, this study was undertaken to identify this disease and to build up basic information about the causal agent. The following aspects of the disease and the causal agent were, therefore studied.

- Symptoms produced by the virus in ginger plants.
- Transmission of the virus through vegetative propagation material and by means of mechanical transmission.
- Changes in the host metabolism due to the viral infection, the 'Pathophysiological studies'.
- Immunological and molecular diagnosis of the virus and its characterization.
- Management of the disease using botanicals, chemicals and of microbial origin.

Review of Literature

2. REVIEW OF LITERATURE

2.1. Symptomatology

Rayachaudhuri and Ganguli (1965) observed severe mosaic symptoms in large cardamom (*Amomum subulatum* Roxb.) leaves. They also observed the gradual coalescing and drying up of the leaves. The flowering in the diseased plants was also found to be reduced. They reported that only one to five flowers developed in the inflorescence of infected plants against 16 to 20 flowers in healthy ones.

A mosaic disease in ginger was reported by Nambiar and Sarma (1974) in which the leaves were appeared with chlorotic flecks. Those flecks were later observed to be developing into slender, spindle shaped, discontinuous chlorotic streaks. They also observed that these streaks to be running parallel to the veins and to each other. The diseased leaves were not malformed and they were similar to healthy leaves in size and shape. Some cases were reported with large patches of light and dark regions in the leaves.

Yellowish and dark green mosaic symptoms on the leaves of ginger were reported by So (1980) from the plantations in Korea. The leaves of infected plants also exhibited chlorotic streaks. The general, leaf as well as rhizome stunting of the infected ginger plants was also reported by him. He observed about 43% incidence in the fields. He also reported that the disease could appear in the plants at both early as well as later stages.

Thin, slender, spindle shaped chlorotic flecks were reported by Thomas (1986) in ginger plants of Australia and he called it as *Ginger chlorotic fleck virus* (GCFV). He observed prominent chlorotic flecks 1-10 mm long. The flecks were running paerallel to the veins and they were centred on the veins. He also observed that younger leaves were developing symptoms first and there were no obvious symptoms on the rhizomes of the infected plants.

Siljo *et al.*(2012) reported a novel kind of viral disease symptom in cardamom, showing chlorotic streaks on veins. They diagnosed the disease with continuous or discontinuous chlorotic streaks along the veins and midrib. Based on these symptoms observed, they called it as 'Chlorotic streak' in cardamom.

Zhang *et al.* (2016) observed mosaic, streaking and severe cupping of leaves with browning of flowers in flowering ginger (*Alpinia purpurata*) plants in Hawaii.

2.2. Transmission

Nambiar and Sarma (1974) reported that the mosaic disease of ginger could effectively be transmitted through infected rhizomes. They observed clear mosaic symptoms on newly emerging leaves of rhizomes which were collected from the virus- infected mother plants. They also conducted studies on sap transmission of the virus. The virus was mechanically inoculated onto ginger, *Nicotiana tabacum* var. *xanthi*, *N. tabacum* var. *rustica*, *N. tabacum* var. *harison special*, *N. glutinosa*, *Elettaria cardamomum*, *Curcuma longa* and *C. aromatica* and none of them responded positively.

The ability of artificial inoculation by sap transmission of the mosaic disease of ginger was reported by So (1980). He also stated that the virus was transmissible to 23 other species of plants, which were known to be susceptible to *Cucumber mosaic virus* (CMV) upon mechanical transmission. Among those, *Chenopodium amaranticolor*, *Nicotiana tabaccum* var. Havana, cowpea, cucumber and tomato were the major species of plants. These plants were reported to express the symptoms by him, upon mechanical inoculation.

Low efficiency mechanical transmission of *Ginger chlorotic fleck virus* (GCFV) was observed by Thomas (1986). He noticed that two plants became infected from a total of six inoculated. He also added that the transmission efficiency increased with the concentration of virus inoculum and the GCFV was not transmitted by the aphids *Myzus persicae*, *Pentalonia nigronervosa*, *Rhophalosiphum maidis* or *R. padi*. The major indicator host plants which he

tested for the local lesions viz. *Chenopodium amaranticolor, C. quinoa, Cucumis sativus, Datura strammonium* gave no symptoms nor local lesions upon inoculation.

Magnaye and Epsino (1990) and Rodoni *et al.* (1999) found that the *Banana bract mosaic virus* (Genus *Potyvirus*) transmission is primarily through the infected suckers and secondarily by means of the banana aphid vector, *P. nigronervosa*. Fan *et al.* (1999) stated that a virus isolate from ginger could infect 25 plant species from five families when inoculated mechanically on to 43 plant species from 13 families tested in China.

Liou *et al.* (2003) reported *Alpinia mosaic virus* (AlpMV), a new virus known to infect plants of Zigiberaceae family was transmissible only by the banana aphid vector, *P. nigronervosa* in a non- persistent manner. Zhang *et al.* (2016) used leaf extracts of BBrMV- infected *A. purpurata* and done mechanical inoculation to the leaves of *Cassia occidentalis, Chenopodium amaranticolor, Brassica campestris, Capsella rubella, Solanum lycopersicanum, Cucumis sativus, Vigna unguiculata, Phaseolus vulgaris* and *Alpinia purpurata* and observed no symptoms on them. They also couldn't transmit BBrMV to edible ginger by both mechanical and aphid transmission.

Two new begomoviruses infecting on *Macroptilium lathyroides*, a perennial weed in the Caribbean region and Central America and on common bean were described by Idris *et al.*, (2003). The experimental mechanical inoculation gave the expression of mosaic symptoms in *M. lathyroides* eight days after inoculation.

Usharani *et al.*, (2004) observed severe leaf curl disease in potato crop in northern India. The immunological and electron microscopic studies revealed that it was a begomovirus which caused the disease. The virus was described to be a strain of *Tomato leaf curl New Delhi virus* (ToLCNDV). They also reported that the begomovirus from a severely affected potato plant was sap-transmissible onto *Nicotiana benthamiana* seedlings. The plants which were inoculated with the

virus exhibited leaf distortion and stunting after 15 days of inoculation. The mechanical inoculation was done in meristem-derived plantlets of potato cv. Kufri Anand and they also gave same results.

Tomato leaf curl disease was observed in 1981 for the first time in Taiwan. In electron microscopy it was identified as geminivirus particles and as *Tomato leaf curl Taiwan virus* (ToLCTWV) by DNA sequencing by Tsai *et al*., (2011). They tested the efficiency of mechanical transmission of the virus in tomato and *Nicotiana benthamiana*. From the test, mechanical transmissibility was confirmed only from plants co-infected by ToLCTWV DNA-A and DNA-B.

The efficiency of mechanical transmission of ToLCNDV to cucurbit germplasm was studied by Lòpez *et al*., (2015). ToLCNDV was inoculated mechanically on different cucurbitaceae taxa. They assayed 99 accessions from 33 different countries, which comprises of four genera and 13 species for validating the efficiency of mechanical transmission. ToLCNDV was efficiently inoculated in all the four genera assayed: *Cucumis*, *Cucurbita*, *Citrullus* and *Lagenaria*.

2.3. Host – Pathogen interaction

2.3.1. Carbohydrates

Ramiah (1978) found that there was decrease in synthesis of total carbohydrates in infected leaves of susceptible cowpea. Singh and Singh (1980) reported a decrease of total sugar and starch in leaf tissues of sunhemp infected with *Bean mosaic virus*. Singh and Singh (1984) observed that the virus infection decreased the total sugar and starch in cowpea cultivars infected with *Southern bean mosaic virus* and *Cowpea mosaic virus*.

Johri and Pandhi (1985) reported that the carbohydrate level declined positively with severity of disease symptoms in case of yellow vein mosaic of okra. Sastry and Nayudu (1988) reported a higher quantity of carbohydrate in hypersensitive cowpea cultivars infected with tobacco ringspot NEPO virus and suggested that the infected area could act as a metabolic sink. Bensal *et al.* (1990)

found that there was significant increase in amount of total sugars, amino acid and phenol in virus infected plants sprayed with sorghum leaf extract.

Sarma *et al.* (1995) reported reduction in reducing sugars and increase in total sugar and non-reducing sugar contents in bhendi due to BYVMV infection. Thind *et al.* (1996) reported that the amount of total sugars and starch decreased in black gram infected with *Yellow mosaic virus* when compared with healthy plants. Bhagat and Yadav (1997) reported that healthy leaves of susceptible cultivars showed higher content of total sugar than resistant one in the case of *Bhendi yellow vein mosaic virus* infected plants. The increased sugar content was attributed to their accumulation, as a result of the disruption of normal phloem transport.

Banerjee and Kalloo (1998) recorded lower amount of reducing sugars and higher amount of non-reducing sugars in susceptible varieties to TLCV as compared to highly resistant *L. esculentum* fsp. *glabratum* B6013 and resistant *L. pimpinellifolium* A1921 lines. Sutha *et al.* (1998 a) studied the changes in concentrations of chemical constituents in tomato caused by TSWV infection and revealed that there was accumulation of carbohydrate in infected plants. Total sugars decreased in infected leaves and the reduction was more in the initial stages compared to later stages of infection. In contrast to sugar concentration, the starch increased in infected plants at all stages of infection. Sutha *et al.* (1998 b) reported that TSWV infection reduced the concentration of total, reducing and non-reducing sugars of tomato fruits.

Mali *et al.* (2000) reported that *Mungbean yellow mosaic virus* (MYMV) infection on moth bean resulted in significant reduction of total soluble carbohydrates in susceptible cultivar when compared to resistant cultivar. Sindhu (2001) also reported a reduction in the level of carbohydrate content in cowpea plants inoculated with BlCMV compared to healthy control. Raghavendra (2002) reported post infection decrease in reducing and non-reducing sugars and total sugars in tomato plants infected with TLCV.

Sugarcane plants infected with *Sugarcane yellow leaf virus* showed abnormal starch accumulation in mesophyll and bundle sheath cells compared to healthy plants (Yan *et al.*, 2008). Total sugar content was increased in CMV infected tapioca leaves showing an increase of 12.8% (Philip, 2010). Sinha and Srivastava (2010) observed increase in sugar and starch contents in finger millet plants infected with mottle streak virus. A significant reduction in total sugar content in BlCMV infected cowpea plants was observed by Krishnapriya (2015).

2.3.2. Chlorophyll

Ramakrishnan *et al.* (1968) reported that in both healthy as well as diseased cassava plants, chlorophyll a and b increased with age. But, these were considerably lower in diseased plants than in healthy plants from early stages. Alagianagalingam and Ramakrishnan (1979) observed a reduction in carotene and chlorophyll contents in cassava leaves infected by *Cassava mosaic virus,* as compared to healthy leaves. Ayanru and Sharma (1982) also reported the similar reduction of chlorophyll a and b in mosaic virus infected cassava plants.

Sarma *et al.* (1995) reported that BYVMV infection reduced the chlorophyll content (chlorophyll a, b and total chlorophyll) of bhendi leaves. Thind *et al.* (1996) reported decline in the chlorophyll content of mung bean plants infected with yellow mosaic virus, with respect to healthy plants.

Vasanthi and Shanmugam (2003) observed lowest content of chlorophyll a, b and total chlorophyll in ICMV infected sett propagated cassava as compared to meristem derived cassava regenerants. Total chlorophyll, chlorophyll a and chlorophyll b were found to be lower in mung bean plants infected by *Mungbean yellow mosaic virus* (MYMV) (Momol and Pernezny, 2006.).

Arpita and Subrata (2008) investigated changes in biochemical components in mesta plants due to infection with yellow mosaic virus and observed a gradual fall in green pigments like chlorophyll a, b and total chlorophyll at different stages of pathogenesis. Cassava mosaic geminiviruses (CMG) induced physio-biochemical changes in tapioca were investigated by

Philip (2010). He reported a reduction in chlorophyll a and b in infected leaves compared to healthy leaves.

Saveetha *et al.* (2010) recorded that chlorophyll pigments a and b as well as total chlorophyll were reduced due to mottle streak virus in finger millet plants.

2.3.3. Phenol

Ramiah (1978) from his studies on phenol content between healthy and inoculated leaves of cowpea reported that there was no difference in phenol content between them. Sharma *et al.* (1984) studied the effect of viral and fungal infections in musk melon and showed an increasing trend of enzyme activity and phenol component as compared to healthy control, irrespective of the nature of infection.

Rathi *et al.* (1986) assayed total phenols and other biochemical parameters in sterility mosaic resistant and susceptible pigeon pea cultivars. They reported that there was no difference between varieties with respect to total phenol content. Sohal and Bajaj (1993) reported an increase in total phenols in both resistant and susceptible varieties of black gram infected with *Yellow mosaic virus*. Sarma *et al.* (1995) reported an increase in total phenol content of BYVMV infected leaves. Thind *et al.* (1996) recorded an increase in total phenols in the plants infected with yellow mosaic virus as compared to healthy plants due to virus infection.

Sutha *et al.* (1998a) found that both total phenol and *ortho-* dihydroxy phenol increased in *Tomato spotted wilt virus* (TSWV) infected plants. Banerjee and Kalloo (1998) recorded high phenol content in highly resistant *L. esculentum* fsp. *glabratum* B6013 and resistant *L. pimpinellifolium* A1921 lines to TLCV as compared to susceptible varieties. Higher content of phenol and flavanol due to *Cotton leaf curl virus* infection was reported in resistant cultivars with respect to susceptible varieties (Kaur *et al.*, 1998).

Mali *et al.* (2000) reported that OD-phenol was higher in healthy leaves than diseased leaves in case of yellow mosaic virus infected moth bean.

Raghavendra (2002) reported post infection increase in phenols (34.21%) in the plants infected with TLCV. Vasanthy and Shanmugam (2003) observed lowest contents of total phenol and OD-Phenol in healthy sett-propagated cassava as compared to ICMV infected sett-propagated and meristem derived cassava regenerants.

Parashar and Lodha (2007) concluded that the resistance in *Foeniculum vulgare* against Ramularia blight was attributed to the presence of high amount of phenol. Veena (2007) showed a multifold increase in the phenolic content of AVP treated cowpea plants compared to healthy and CABMV inoculated cowpea plants. Meena *et al.* (2008) stated that the phenol synthesizing pathways are accelerated after the pathogen attack and it results in the increased levels of phenols. Saveetha *et al.* (2010) while studying physiological alterations due to viral infection observed increased phenol contents in *Eleusine coracana* (finger millet) plants infected by *Mottle streak virus*.

2.3.4. Protein content

Johri and Pandhi (1985) reported that the total protein content in okra declined in diseased tissues due to *Yellow vein mosaic virus* infection. They also found that the insoluble fraction of protein was increased in diseased tissues as against soluble proteins. Ahmed *et al.* (1992) studied the effect of yellow vein mosaic disease on the physiology of okra and reported that the total protein and soluble proteins were high in virus – free resistant varieties of okra.

Sarma *et al.* (1995) reported that the protein contents increased in bhendi leaves infecred with BYVMV. The extent of increase or decrease of these constituents varied with the different stages of plant growth and in green fruits, the protein content decreased by virus infection. Thind *et al.* (1996) reported that *Mungbean yellow mosaic virus* (MYMV) infection on mungbean lowered the protein content in the leaves. Banerjee and Kalloo (1998) recorded high crude protein content in highly resistant *L. esculentum* fsp. *glabratum* B6013 and

resistant *L. pimpinellifolium* A1921 lines to TLCV as compared to susceptible varieties.

Mali *et al.* (2000) reported that free amino acids and soluble protein content increased with increasing levels of *Yellow mosaic virus* infection in susceptible variety of moth bean. The leaves of *Bean yellow mosaic virus* (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). Protein content was found to be higher in alfalfa plants infected with *Alfalfa mosaic virus* (Yardimci *et al.*, 2006).

Taiwo *et al.* (2007) demonstrated that the protein content was lowered in all the cowpea cultivars and lines as a result of individual as well as mixed infection of *Cowpea aphid borne mosaic virus* (CABMV), *Cowpea mottle virus* (CPMoV) and *Southern bean mosaic virus* (SBMV). There was a reduction from 24.8 to 28.9 per cent in the plants. Veena (2007) studied the biochemical changes due to CABMV infection in cowpea and the total soluble protein was reported higher in case of inoculated plants.

Arpita and Subrata (2008) reported lower protein contents in mesta plants infected with yellow vein mosaic compared to control. Chatterjee and Ghosh (2008) stated that yellow vein disease of mesta cause a reduction in total soluble protein. Ashfaq *et al.* (2010) reported that *Urdbean leaf crinkle* virus (ULCV) infected plants appeared to have increased total soluble protein contents.

Cassava *mosaic virus* (CsMV) infection in cassava resulted in the reduction of total soluble protein content in cassava plants (Philip, 2010). Saveetha *et al.* (2010) reported increased soluble protein contents in finger millet plants due to virus infection. Sinha and Srivastava (2010) also reported increased protein contents in mung bean plants infected by MYMV. Alex (2017) reported that there was a progressive increase in the total protein content in treated, virus inoculated and healthy plants with increase in the age of plants.

2.3.5. Defence related enzymes

Khatri and Chenulu (1970) studied the changes in the peroxidase enzyme activity in leaves of resistant and susceptible cowpea varieties and observed that the peroxidase activity increased in both, but significantly higher in susceptible variety. Ramiah *et al.* (1978) reported that peroxidase (PO) activity increased in the leaves of BYVMV infected plants.

Alagianagalingam and Ramakrishnan (1974) noted significant difference in PO activity in cassava leaves infected with CMG. Batra and Kuhn (1975) found that when primary leaves of hypersensitive soybean plants were infected with *Cowpea chlorotic mottle virus*, the enzymes polyphenol oxidase and peroxidase increased 2-3 times over healthy plants. They also found that the increase was concomitant with the development of acquired resistance.

Wagih and Coutts (1982) reported that *Tobacco necrosis virus* infected cowpea and cucumber showed increase in the amount of extractable peroxidase and polyphenol oxidase activity. Sharma *et al.* (1984) showed the effect of viral and fungal infections in musk melon and reported an increasing trend of enzyme activity when compared to healthy control. Verma and Prasad (1984) reported that spraying aqueous leaf extract of *Clerodendron aculeatum* induced increased activity of catalase, peroxidase and polyphenol oxidase in cluster beans and prevented the infection of sun hemp rosette virus on them.

Rathi *et al.* (1986) assayed peroxidase, polyphenol oxidase and isozyme of peroxidise in pigeon pea resistant and susceptible cultivars to sterility mosaic virus and noted that there was not much difference two varieties with respect to peroxidase and polyphenol oxidase activities. Resistance was characterized by the presence of specific isoperoxidase and proteins. Zaidi *et al.* (1992) reported the changes in phenolic content and phenylalanine ammonia lyase in response to infection by *Carnation etch ring virus*.

Ahmed *et al.* (1992) found that peroxidase and polyphenol oxidase showed no significant difference in virus free susceptible and resistant plants

while studying basis of resistance to yellow vein mosaic virus in okra. Verma *et al.* (1996) reported that the pre inoculation application of AVPs challenged with plant viruses in different hosts results in the activation of key enzymes like peroxidase, polyphenol oxidase, phenylalanine ammonia lyase chitinase and glucanase leading to the suppression of viral pathogen.

Mali *et al.* (2000) reported that the activity of catalase, peroxidase and nitrate reductase was found to reduce with increased intensity of disease in the case of yellow mosaic disease of moth bean (*Vigna aconitifolia)*. Sindhu (2001) studied on the changes of defence related enzymes such as peroxidase, polyphenol oxidase and phenylalanine ammonia lyase and stated that there was significant increase in activities of these enzymes in inoculated cowpea plants.

Clarke *et al.* (2002) and Karthikeyan *et al.* (2007) also observed significant increase in PO activity in *Phaseolus vulgaris* and *Vigna mungo* after *White clover mosaic virus* (WClMV) inoculation and *Urdbean leaf crinkle virus* (ULCV) inoculation, respectively. Vasanthi and Shanmugam (2003) showed increased activities of PO and PAL in meristem derived cassava regenerants compared with enzyme activities in healthy and infected sett propagated cassava.

Bhatia *et al.* (2004) found that the activities of enzymes superoxide dismutase and peroxidase were increased as against decreased activities of catalase in TMV infected tobacco leaves. Veena (2007) observed an increase in defence related enzymes *viz.* peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) in AVP treated cowpea plants challenged with BlCMV compared to that of the healthy and virus inoculated cowpea plants.

Meena *et al.* (2008) reported that PPO was responsible for oxidation of OD-Phenols and observed high polyphenol and lower orthodihydroxy phenol activity in geminivirus infected chilly leaf as compared to healthy, which was responsible for phenol accumulation as oxidation of OD-Phenol was due to this enzyme. Ashfaq *et al.*(2010) reported higher peroxidase activity in healthy leaves

of susceptible genotypes than in resistant genotypes of urd bean inoculated with ULCV.

Solanum lycopersicanum exhibited more PO activity in the presence of TLCV and the activity of the enzyme was found higher in mature than in juvenile leaves (Dieng *et al.*, 2011). Krishnapriya (2015) reported a higher peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) activity for BlCMV inoculated cowpea plants compared to healthy plants.

2.4. Immunological and molecular diagnosis

Rayachaudhuri and Ganguly (1965) conducted rapid chloroplast agglutination tests to locate chirke virus- infected cardamom plants under field conditions. Using this technique, it was possible to detect and rogue out the infected plants from the field. Chirke virus was serologically detected by him in cardamom, ginger and wheat plant samples collected from different farmers' fields of Darjeeling district.

2.4.1. *Enzyme Linked Immunosorbent Assay* (ELISA)

So (1980) stated that a virus isolate from ginger plants showing mosaic symptoms gave positive serological reaction with antiserum to *Cucumber mosaic virus* (CMV).

Seghal (1981) said that many properties of the members of the sobemovirus group are serologically unrelated. Thomas (1986) suggested that the newly observed *Ginger chlorotic fleck virus* (GCFV) reflected its affinities to the sobemovirus group. Conforming to the words of Seghal, he failed to positive serological reaction with the antisera of several sobemoviruses.

Thomas (1986) also tested the reaction of purified GCFV with the antisera of plant viruses viz. carnation mottle, cocksfoot mottle, cucumber fruit streak, cucumber mosaic, cymbidium ringspot, elm latent, galinsoga mosaic, glycine mottle, hibiscus chlorotic ringspot, lucerne transient streak, narcissus tip necrosis, pelargonium flower streak, pelargonium ring pattern, pelargonium vein netting,

phleum mottle, red clover necrotic mosaic, saguaro cactus, solanum nodiflorum mottle, southern bean mosaic, sowbane mosaic, tobacco necrosis, turnip crinkle, turnip rosette and velvet tobacco mottle viruses. He got negative reaction with all of the above said antisera.

A virus isolate from ginger in Laiwu, Shandong Province of China was identified serologically matching to *Tobacco mosaic virus* (TMV) by Fan *et al.* (1999).

Jacob and Usha (2001) tested the Indian isolate of CdMV with the antisera of other potyviruses viz. peanut stripe, peanut mottle, pepper vein banding, the Guatemalan isolate of CdMV, bean yellow mosaic, clover yellow vein mosaic, Johnsongrass mosaic, watermelon mosaic viruses and potato virus Y. Of these, only the Guatemalan isolate of CdMV antisera showed positive reaction with the Indian isolate.

Zhang *et al.* (2016) carried out DAS- ELISA in flowering ginger (*Alpinia purpurata*) using the antiserum of *Banana bract mosaic virus* (BBrMV). In the assay they got positive reaction and detected BBrMV in *A. purpurata.*

2.4.2. Molecular detection and characterization

Liou *et al.* (2003) carried out RT- PCR and TA cloning with a cDNA clone corresponding to the 3'- portion of *Alpinia mosaic virus* (AlpMV) genome. From the sequence and phylogenetic analyses, they suggested that the AlpMV has more identity with the genus *Macluravirus*, rather than the present genus *Potyvirus*.

Siljo *et al.* (2012) identified that the chlorotic streak- infected cardamom plants tested negative in RT- PCR for *Cardamom mosaic virus* (CdMV). They did RT- PCR again using the primers targeted to conserved regions of *Potyvirus*. It gave an ~700 bp amplicon, upon sequencing and BLAST analysis showed *Banana bract mosaic virus* (BBrMV) as the most related virus. Cloning and sequencing of the coat protein gene of the virus isolates from different

geographical regions was carried out using primers specific for BBrMV, which gave positive results.

Zhang *et al.* (2016) achieved deep sequencing of BBrMV from flowering ginger (*Alpinia purpurata*) and developed an immunocapture reverse transcription loop- mediated isothermal amplification (IC-RT-LAMP) assay.

2.5. Management of the disease

By spraying a material for the management of a disease, a resistance mechanism is being induced in the plant. According to van Loon *et al.*, "induced resistance is a physiological 'state of enhanced defensive capacity' elicited by specific environmental stimuli, whereby the plant's innate defenses are potentiated against subsequent biotic challenges". This resistance is successful against a broad range of pathogens. Either biotic or abiotic agents can be used for inducing resistance.

2.5.1. Biotic agents

PGPR mediated systemic resistance is frequently associated with inception of defence mechanism including the early and augmented expression of defence enzymes such as chitinase, glucanase, peroxidase and phenylalanine ammonialyase and accretion of phenolics, phytoalexins and lignins (Mosch *et al.*, 1993; Schneider and Ullrich, 1994; Chen *et al.*, 2000; Nandakumar *et al.*, 2001).

Pseudomonas fluorescens, a root colonizing bacterium was evaluated against the leision inducing *Tobacco necrosis virus* (TNV) in tobacco, by Maurhofer *et al* (1994). The *P. fluorescens* treated plants were observed with reduced TNV induced lesions. They reported that induced systemic resistance (ISR) by *P. Fluorescens* strain CHA0 against TNV in tobacco was coupled with accumulation of PR proteins namely β-1,3 glucanase and endo chitinases.

Induced systemic resistance (ISR) elicited by plant growth promoting rhizobacteria (PGPR) has shown promise in managing a wide spectrum of plant pathogens, including virus, in several plant species under greenhouse and field

environments (Murphy *et al*., 2000; 2003; Raupach *et al*., 1996; Wei *et al*., 1996; Zehnder *et al*., 2000). Murphy *et al*. (2000) evaluated the effect of PGPR under field conditions for induced resistance to *Tomato mottle virus* (ToMoV). They revealed that under natural conditions of high levels of vector- virus pressure, some PGPR treatments resulted in reduced ToMoV disease severity.

Zehnder *et al*. (2000) studied specific strains of PGPR for induced resistance against *Cucumber mosaic virus* (CMV) in tomato. They identified PGPR strains that protected tomato from the systemic infection by CMV, both under greenhouse and field conditions. In greenhouse experiments, the plants were mechanically inoculated with CMV and the percentage of symptomatic plants in the most effective PGPR treatments ranged from 32 to 58 per cent in contrast with the 88 to 98 per cent of the control treatment.

Kanchalee *et al*. (2003) stated that four mixtures of PGPR strains elicited ISR in several plants against diverse plant pathogens. They evaluated these mixtures against CMV and observed decline in disease incidence. Ryu *et al*. (2004) found that *Arabidopsis thaliana* ecotype Columbia plants (Col-0) treated with PGPR, *Serratia marcescenes* strain 90-166 and *Bacillus pumilus* strain SE34 had appreciably reduced symptom severity of CMV infection.

2.5.2. Abiotic agents

2.5.2.1. Botanicals

Neem seed oil and neem leaf extracts had been reported to inhibit lesion production by mechanically transmitted viruses when mixed with the inoculum or when applied to test plants (Verma, 1974; Choudhuri and Saha, 1985; Zaidi *et al*., 1988).

Aiyanathan and Narayanaswamy (1988) studied the effect of neem oil on rice tungro virus infection. They observed that the pre-inoculation as well as post-inoculation sprays of neem oil (5%) reduced RTV infection. Mariappan *et al*.

(1988) recorded significant reduction in the transmission of rice tungro virus by *Nephotettix virescens* in neem oil-sprayed plants.

The local lesion production by TMV was found declined on *Nicotiana glutinosa, N. tabaccum* var. *samsun, Chenopodium amaranticolor* and *Datura stramonium* pretreated with neem oil (Roychoudhuri and Jain, 1993).

Verma and Kumar (1980) reported that the leaf extracts of *Datura* spp., *Azadirachta indica* and *Mirabilis jalapa* contained antiviral proteins which repressed infection by CMV, TMV and yellow vein mosaic virus in black gram. Sreelekha (1987) revealed that cowpea mosaic infection could be effectively controlled by pre inoculation spraying of *Bougainvillea* spp. and *Eupatorium odoratum*. Habuka *et al.* (1991) found that antiviral principles isolated from roots of *Mirabilis jalapa* inhibited mechanical transmission of plant viruses. Balasaraswathy *et al.* (1998) from their studies, suggested that foliar spray of leaf extract of *Bougainvillea spectabilis* was effective in inhibiting infection of TSWV, TMV, CMV and CaMV.

Vivanco *et al.* (1999) evaluated extracts of *Mirabilis jalapa* containing a ribosome inactivating protein (RIP) called mirabilis antiviral protein (MAP), against infection by *Potato virus X, Potato virus Y, Potato leaf roll virus* and *Potato spindle tuber viroid.* They found there were noteworthy reductions in viral incidence.

Bhatia *et al.* (2004) evaluated the antiviral proteins from *Bougainvillea* along with virus and also before virus inoculation. When AVPs were applied prior to virus inoculation or in combination with virus, there was suppression of necrotic lesion formation in local lesion host. Renukadevi *et al.* (2004) reported that the AVP from *M. jalapa* (MAP) and *Herpula cupanioides* (HAP) were highly effective in inhibiting TSWV. Pre-application of MAP and HAP induced the activity of phenols, peroxidise (PO), polyphenol oxidase (PPO) and phenylalanine ammonialyase (PAL) leading to the suppression of TSWV on local lesion and systemic host.

Prasad *et al.* (2007) evaluated the efficacy of certain plant extracts in reducing *Bean common mosaic potyvirus* strain *Blackeye cowpea mosaic* disease (BCMV-BlCMV) in cowpea. They mixed the viral inoculums with these plant extracts and inoculated to young seedlings. From this they reported that *B. spectabilis*, *Canthium inerme* and *M. jalapa* extracts reduced the disease incidence upto 42, 40 and 48 per cent respectively under greenhouse environment when compared to control.

2.5.2.2. Chemicals having antiviral properties

White (1979) reported that pathogenesis related (PR) protein accumulation and resistance to Tobacco mosaic virus (TMV) could be induced by treatment of tobacco with salicylic acid (SA), aspirin (acetyl SA or ASA) or benzoic acid (BA). Conti *et al.* (1988) stated that a very low concentration of 0.01 per cent equivalent to 50 µM ASA was effective in inducing resistance in *Datura* against TMV. The effect was greater when ASA was applied after the inoculation of virus.

Treatment with SA caused reduced accumulation of TMV RNA in directly inoculated TMV-susceptible tobacco leaf tissue (Chivasa *et al.*, 1997). In SA-treated tobacco, the accumulation of at least two viruses, TMV and *Potato virus X* (PVX) was inhibited at the site of inoculation (Chivasa *et al.*, 1997; Naylor *et al.*, 1998). Dempsey *et al.* (1999) and Durrant and Dong (2004) stated that salicylic acid plays a critical signalling role in the activation of disease resistance in plants.

Murphy *et al.* (2001) observed that a state of enhanced disease resistance can be induced by treatment with solutions of SA or its synthetic functional analogues. Murphy and Carr (2002) reported that the replication of TMV was greatly decreased in the leaf mesophyll cells of SA-treated plants. They also added that it induced resistance of movement of virus between the epidermal cells of host plant. Wong *et al.* (2002) found that SA, non-lethal concentrations of cyanide and antimycin A (AA) could induce resistance to *Turnip vein clearing virus* (TVCV). They observed inhibition of TVCV in SA-treated *Arabidopsis*.

Gruner *et al.* (2003) reported that PR-1a is induced in tobacco plants during the hypersensitive response (HR) after exposure of plants to SA. Singh *et al.* (2004) reported multiple antiviral defence mechanism of SA. They observed that SA triggers resistance to viral infection process such as replication, cell to cell movement and long distance movement.

Carl *et al.* (2005) found that SA- induced resistance against CMV in tobacco results from inhibition of systemic virus movement. They also found that inhibition of CMV systemic movement is also induced by SA and antimycin A in *Arabidopsis thaliana*.

White *et al.* (1986) reported that the chemicals manganese chloride and barium chloride induced resistance in Xanthi-nc tobacco leaves to TMV infection. Bioassy of chemicals such as SA, manganese chloride and barium chloride to evaluate their efficiency in reducing the symptoms caused by *Cowpea aphid borne mosaic virus* (CABMV) in *Chenopodium amaranticolor* and cowpea revealed that the post-inoculation treatment of manganese chloride gave maximum inhibition of symptoms (Radhika, 1999).

Pun *et al.* (2000) reported that barium chloride was most effective in reducing the symptoms of *Bhindi yellow vein mosaic virus* (BYVMV) when sprayed exogenously on bhendi plants.

Materials and Methods

3. MATERIALS AND METHODS

The present research work entitled 'Etiology and management of mosaic disease in ginger (*Zingiber officinale* Rosc.)' was carried out in the College of Agriculture, Vellayani during 2015-2017, with the objectives of identifying and characterizing the virus and for the manangement of the virus.

3.1. SYMPTOMATOLOGY

Ginger plants of variety Karthika infected with the mosaic virus were found in Instructional Farm, College of Agriculture, Vellayani. The rhizomes of the infected plants were purchased and they were planted for germination and observed for the expression of the symptoms. The development of symptoms were observed at weekly interval upto 120 days after planting. These plants were used as a source of inoculum of the virus for further studies.

3.2. TRANSMISSION

3.2.1. *Mechanical transmission*

Virus- infected ginger plants were used as the source of inoculum for mechanical transmission. Sap transmission was performed using 0.1 *M* Sodium phosphate buffer (pH 7.0) (Appendix I). Carborandum powder was used as abrasive for all the sap transmission studies. The young leaves of ginger plants which showed clear mosaic symptoms were selected for sap extraction. One part of the leaf tissue was homogenized with 1.5 parts of the phosphate buffer in a chilled mortar and pestle. The homogenate was maintained in an ice box and immediately used for inoculation. Carborandum powder was uniformly dusted over the leaves of ginger plants selected for inoculation, prior to inoculation. Inoculation was done by gently rubbing the upper surface of the freshly emerged two to three leaves using finger dipped in the inoculum. Immediately after the inoculated surface has dried, it was gently rinsed off with clear distilled water using a wash bottle. The development of these symptoms on these plants was recorded up to 120 days.

3.2.2. *Transmission through planting material*

Rhizomes, the planting material for ginger were also used for conducting transmission studies. Infected and healthy plants were used for it. Both were planted in pots separately and examined for the development of symptoms upon germination.

3.3. BIOCHEMICAL ANALYSIS

Biochemical analyses of healthy and inoculated ginger plants were conducted. Mechanical inoculation was given to the newly formed leaves of ginger after germination. Samples were taken at intervals of 30, 60, 90 and 120 days after mechanical inoculation.

Biochemical analyses were conducted to estimate the changes in total carbohydrates, chlorophyll, phenol and protein. Analysis of defence related enzymes such as peroxidase, polyphenol oxidase and phenylalanine ammonia-lyase were also carried out. Statistical analysis was done using completely randomized design (CRD). Protein profile study was conducted using SDS-PAGE. Isozyme analysis of diseased and healthy plants was performed in native PAGE.

3.3.1. Estimation of total carbohydrates

The determination of total carbohydrates was done through Anthrone method (Hedge and Hofreiter, 1962). In this procedure, 100 mg of the sample was taken in a boiling tube and it was subjected to hydrolysis by keeping in a boiling water bath for three hours with 5 ml of 2.5 N HCl. After this it was cooled down to room temperature. Solid sodium carbonate was added into the tube for neutralisation until the effervescence ceased and it was made upto 100 ml. The samples were centrifuged at 5000 rpm for 15 minutes at 4°C. From this the supernatant was collected and 0.5 ml was taken as aliquot for analysis. The aliquot was made upto 1 ml using distilled water and 4 ml of anthrone reagent was added into it. The mixture was heated for 8 minutes in a boiling water bath. After heating

it was cooled down rapidly and the green- dark green colour was read at 630 nm in a spectrophotometer (Eppendorf Bio Spectrophotometer).

3.3.2. Estimation of chlorophyll content

For chlorophyll estimation, 1 g of freshly cut and well mixed representative samples of leaves were taken. It was ground to a fine pulp with addition of 20 ml of 80% chilled acetone. It was centrifuged at 5000 rpm for five minutes at 4°C and the supernatant was transferred into a 100 ml volumetric flask. The residue was again ground in 20 ml of 80% chilled acetone, centrifuged as earlier and the supernatant was transferred to the same volumetric flask. This procedure was repeated until the residue became colourless. The mortar and pestle were washed thoroughly with 80% acetone and the clear washings were collected in the volumetric flask. The volume was made upto 100 ml using 80% acetone. The absorbance values were read at 645 and 663 nm in a spectrophotometer against the blank, 80% acetone. Chlorophyll a, chlorophyll b and total chlorophyll contents were calculated using the following formulae:

mg chlorophyll per gram tissue:

Chlorophyll a: $[12.7 (A\ 663) - 2.69 (A\ 645)] \times \frac{v}{1000w}$

Chlorophyll b: $[22.9 (A\ 645) - 4.68 (A\ 663)] \times \frac{v}{1000w}$

Total chlorophyll: $[20.2(A\ 645) + 8.02 (A\ 663)] \times \frac{v}{1000w}$

Where, A = absorbance at specific wavelength,

v = final volume of the chlorophyll extract and

w = fresh weight of the tissue extracted.

3.3.3. Estimation of phenol

Phenol content was estimated following the procedure described by Bray and Thorpe in 1954. For the estimation, 1 g leaf sample was ground in 10 ml of 80% ethanol. The homogenate was centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant was saved and residue was extracted with five times volume of ethanol and centrifuged. The supernatant was saved and evaporated to dryness in boiling water bath. The residue was dissolved in 5 ml distilled water. An aliquot of 0.3 ml was taken and made up to 3 ml with distilled water. To this, 0.5 ml of Folin – Ciocalteau (FC) reagent was added and 2 ml of 20% sodium carbonate solution was added to it after three minutes. This was mixed thoroughly and kept in boiling water for one minute. The reaction mixture was cooled the absorbance was measured at 650 nm against blank (3 ml distilled water, 0.5 ml FC and 2 ml of 20% sodium carbonate).

3.3.4. Estimation of soluble proteins

The soluble protein estimation was done by following procedure described by Bradford (1976). In this method, 1 g leaf sample was homogenized in 10 ml of 0.1 M sodium acetate buffer (pH- 4.7) (Appendix II) and centrifuged at 5000 rpm for 15 minutes at 4°C (Eppendorf Centrifuge 5804 R). The supernatant was saved for the estimation of soluble protein. The reaction mixture was constituted with 0.5 ml enzyme of extract, 0.5 ml of distilled water and 5 ml of dilute dye solution (5X dilution of Coomassie Brilliant Blue G250). Absorbance was read at 595 nm against reagent blank which constituted 1 ml of distilled water and 5 ml of diluted dye solution. Bovine serum albumin was used as the protein standard and the protein content was expressed as mg albumin equivalent of soluble protein per gram (on fresh weight basis). The absorbance was read in Eppendorf Bio Spectrophotometer.

3.3.5. Estimation of defence related enzymes

3.3.5.1. *Estimation of peroxidise (PO)*

Peroxidise activity was estimated by spectrophotometric method described by Srivastava (1987). For this, 1 g of leaf sample was homogenized in 5 ml of 0.1 M sodium phosphate buffer (pH- 6.5) (Appendix III), to which a pinch of polyvinyl pyrrolidone (PVP) was added. The homogenate was centrifuged at 5000 rpm for 15 minutes at 4°C (Eppendorf Centrifuge 5804 R). The supernatant was saved and was used as the enzyme extract. The reaction mixture consisted of 1 ml of 0.05 M pyrrogallol and 50 µl of enzyme extract. To this, 1 ml of 1% hydrogen peroxide was added for initiating the reaction. Readings were taken at 420 nm, in intervals of 30 s, up to 180 s. PO activity was expressed as changes in absorbance per min per gram fresh weight of tissue.

3.3.5.2. *Estimation of polyphenol oxidase (PPO)*

The method described by Mayer *et al* (1965). Was followed for the estimation of PPO activity. The procedure for enzyme extract preparation was as same as that for PO. The reaction mixture consisted of 1 ml of 0.1 M sodium phosphate buffer (pH- 6.5) and 50 µl of enzyme extract. For the reaction initiation, 1 ml of 0.01 M pyrrocatechol was added to the reaction mixture and the readings were taken in 30 s interval up to 180 s, at 495 nm. The PPO activity was expressed as change in absorbance of the reaction mixture per min per gram on fresh weight basis.

3.3.5.3. *Estimation of phenylalanine ammonialyase (PAL)*

The estimation of PAL activity was carried out by the procedure described by Dickerson *et al.*(1984). In this method, 1 g of leaf sample was homogenized in 5 ml of 0.1 M sodium borate buffer (pH- 8.8) (Appendix III), to which a pinch of PVP was added. The homogenate was centrifuged at 10,000 rpm for 10 minutes at 4°C (Eppendorf Centrifuge 5804 R). The supernatant was used for the assay of PAL. The reaction mixture consisted of 3 ml of 0.1 M sodium borate buffer, 0.2

ml of enzyme extract and 0.1 ml of 12 mM *L* – Phenylalanine in same buffer. A blank devoid of the reaction enzyme extract was also prepared. The reaction mixture and blank were incubated for 30 min at 40°C. the reaction was stopped by adding 0.2 ml of 3N HCl and the absorbance was read at 290 nm. PAL activity was calculated graphically using cinnamic acid standards and 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 proteins of ginger leaves were carried out as per the procedure described by Laemelli (1970).

One gram each of healthy and inoculated ginger leaves at different stages viz. 30, 60, 90 and 120 DAT were used for the analysis. The leaf samples were homogenized in cold protein denaturing buffer (Appendix V) in the ratio of 1:1.50. the homogenization was done in a chilled mortar and pestle. The homogenate was strained with cotton and subjected to centrifugation at 5000 rpm for 15 min at 4°C in Eppendorf Centrifuge 5804 R. 0.5 ml of the supernatants was transferred separately to other centrifugation tubes. To each of these tubes, 1 ml of chilled acetone was added. This acetone- supernatant mixture was stored at 4°C for 30 min for precipitation of proteins. After precipitation the samples were again centrifuged at 5000 rpm for 10 minutes. The supernatant was discarded and the pellets were retained. This pellet was resuspended in 75 µl protein denaturing buffer and 10 µl of SDS sample buffer (explained in reagents). After thorough mixing using micro pestles, it was again subjected to centrifugation at 5000 rpm for 10 minutes. The supernatants were saved and the pellets were discarded. To the supernatants collected, again 75 µl protein denaturing buffer and 10 µl of SDS sample buffer were added and vortexed thoroughly. After vortexing, the samples were heated for 3 min in a boiling water bath. These heated samples were readily used for loading. Standard was prepared using known molecular weight marker (PMWM, GeNei, Cat No. 105979) 10µl added with 10µl SDS sample buffer.

Reagents

a) Acrylamide stock (30%)

Acrylamide	-	29.20 g
Bis- acrylamide	-	0.80 g
Double distilled water	-	100.00 g

b) Separating (resolving) gel buffer stock (1.5 *M* Tris- HCl, pH: 8.80)

Tris base (18.15 g) was dissolved in approximately 50 ml of double distilled water. The pH was adjusted to 8.80 with 6 *N* HCl and made up the volume to 100 ml with double distilled water and stored at 4°C.

c) Stacking gel buffer stock (0.5 *M* Tris- HCl, pH: 6.80)

Tris base (6.00 g) was dissolved in approximately 60 ml of double distilled water and adjusted the pH to 6.80 with 6 *N* HCl and the volume was made up to 100 ml with double distilled water and stored at 4°C.

d) Polymerising agents

Ammonium persulphate (APS) 10 per cent prepared freshly before use.

Tetramethyl ethylene diamine (TEMED)- Fresh from refrigerator.

e) Electrode buffer (pH 8.30)

Tris base	-	6.00 g
Glycine	-	28.80 g
SDS	-	2.00 g
Double distilled water	-	2 L

f) Sample buffer

Double distilled water	- 2.60 ml
0.5 *M* Tris HCl (pH 6.80)	- 1.00 ml
2-mercaptoethanol	- 0.80 ml
Glycerol	- 1.60 ml
SDS- 20% (w/v)	- 1.60 ml
0.5% Bromophenol blue	- 0.40 ml

g) Staining solution

Coomassie brilliant blue R- 250	- 0.10 g
Methanol	- 40.00 ml
Glacial acetic acid	- 10.00 ml
Double distilled water	- 50.00 ml

h) Destaining solution

As above without Coomassie brilliant blue R- 250

Procedure

Separating gel was casted first, followed by stacking gel by mixing the various components as indicated below:

a)Preparation of separating gel (12%)

Double distilled water	- 6.70 ml
Tris HCl, pH 8.8	- 5.00 ml
SDS 10%	- 0.20 ml

Acrylamide stock - 8.00 ml

The above said components were mixed well and degassed for three minutes and then the following were added immediately:

10% APS -0.10 ml

TEMED - 0.01 ml

The separating gel was mixed well and poured immediately between glass plates and a layer of water was added above to it to hasten the polymerising process.

b) Preparation of stacking gel

Double distilled water - 6.10 ml

Tris HCl, pH 6.8 - 2.50 ml

SDS 10% - 0.20 ml

Acrylamide stock - 1.30 ml

The above said components were mixed well, degassed and the following were supplemented:

APS 10% - 0.05 ml

TEMED - 0.10 ml

The water layered over the resolving gel was removed and the stacking gel was poured over the polymerized resolving gel, and the comb was kept in position.

After the polymerization of stacking gel, the comb was removed carefully and the electrode buffer was filled. Then the standards of known molecular weight and the samples were loaded into the wells. The electrophoresis was performed at 50 V (BIORAD Mini-Protean®Tetra System) till the dye reached the resolving gel. Then the voltage was increased to 60 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 15 minutes with uniform shaking. Then the stain was removed and the gel was immersed in the destaining solution. Timely replacement of destaining solution for several times was done for efficient destaining. The protein appeared as bands in the gel was photographed after placing on a transilluminator (Appliance Model White/ UV TMW- 20).

3.3.7. Electrophoretic analysis of isozyme

Electrophoretic separation of multiple forms of the enzyme peroxidase was undertaken in healthy as well as inoculated ginger leaves.

Peroxidase isozyme analysis

Soluble and ionically bound enzymes were extracted by grinding the sample buffer (Appendix VI) under chilled condition in 50 mM Tris-Cl (pH 7.6) in the ratio of 1:1.5 w/v. The homogenate was centrifuged at 15,000 rpm for 10 minutes at 4°C (Eppendorf Centrifuge 5804 R). the resulting supernatant was used for isozyme analysis. Proteins extracted by 50 mM Tris (pH 7.6) were separated by gel electrophoresis (BIORAD Mini-Protean®Tetra System) in 7.5 per cent gel. The gel was prepared using the stock solution prepared for protein gel electrophoresis without SDS (native gel). Two per cent Triton X-100 was added in 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 minutes at room temperature. The gel was transferred to 0.1 M hydrogen peroxide until visible bands were developed.

3.4. Immunological and molecular diagnosis

The virus isolates from different diseased samples were diagnosed using polyclonal antiserum in enzyme linked immunosorbant assay (ELISA) and dot immunobinding assay (DIBA). The molecular diagnosis using polymerase chain reaction (PCR) was also conducted based on the coat protein gene (CP gene).

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

One gram of leaf sample was homogenized in 5 ml of coating buffer (carbonate buffer) (Appendix IX)containing 2% polyvinyl pyrrolidone (PVP). The homogenization was carried out in chilled condition. The homogenate was centrifuged at 5000 rpm for 10 minutes at 4°C (Eppendorf Centrifuge 5804 R). Samples were loaded at the rate of 100 µl into immunological plates. Three treatments were maintained viz. Healthy, infected and blank. Each treatment was replicated twice. These loaded plates were incubated for two hours at 37°C. After the incubation period, the plate was washed thrice in a microplate washer, each wash at an interval of three minutes. After washing the wells were subjected to blocking with 5% spray- dried milk (SDM), with 100 µl in each well and kept for incubation for two hours at 37°C. Then it was washed as earlier. After washing the plate was treated with 100 µl of polyclonal primary antibody diluted in PBS-TPO, in dilution of 1:200. Then the plates were kept for incubation for one hour as earlier and washed after that. The antibodies against *Tobacco mosaic virus* (TMV), *Cucumber mosaic virus* (CMV), *Sugarcane bacilliform virus* (SCBV), *Southern bean mosaic virus* (SBMV) and *Banana bract mosaic virus* (BBrMV) were used. Later, 100 µl of anti- rabbit immunoglobulin (secondary antibody) diluted in PBS- TPO was loaded in each well of the plate at the rate of 1:10,000 dilution, incubated for two hours and washed as earlier. Then, 100 µl of substrate solution (p-nitrophenyl phosphate) in diethanolamine buffer (1g/ml) was loaded to the wells and incubated for 20-30 minutes at 37°C. after the incubation period, the plate was examined for yellowish colour development. The intensity of the colour was read at 405 nm in an ELISA reader (Microplate Reader 680, BIORAD).

3.4.2. Triple antibody sandwich ELISA (TAS- ELISA)

Sri Lankan cassava mosaic virus (SLCMV), *African cassava mosaic virus*(ACMV) anrtibodies were tested in this procedure. The specific antibody was diluted in coating buffer (1: 1000 dilution) and 200 µl of this was added to each

well of the microtiter plate. Then the plate was covered and incubated at 37°C for 2- 4 hours. After incubation the plate was washed three times with PBS-T at an interval of two minutes between each wash. Later the plate was subjected to blocking, with 200 μl of 2% SDM in PBS-T in each well. The plate was then incubated at 37°C for 30 minutes. Later the blocking solution was removed and the plate was tap dried.

Samples were extracted in extraction buffer (Appendix IX) at 1:10 (w/v) dilution. 200 μl of the test samples were added in three replications to the wells. After this the plate was covered and incubated overnight at 4°C. After the incubation the plate was washed thrice as earlier and 200 μl of monoclonal antibody in conjugate buffer was added to each well. Then the plate was again covered and incubated at 37°C for 2- 4 hours. After washing as done earlier, diluted RAM-AP (1:1000 dilution) in conjugate buffer was added 200 μl in each well. After this the plate was covered and incubated at 37°C for one hour and then washed thrice as earlier. 200 μl aliquotes of freshly prepared substrate (1 mg/ml p-nitrophenyl phosphate) was added to each well. The plate was then covered and incubated at 37°C for 30 minutes, the colour developed was assessed spectrophotometrically in a microplate reader at 405 nm (Microplate Reader 680, BIORAD).

3.4.3. Dot Immunobinding Assay (DIBA)

One gram of plant sample (healthy and diseased) was homogenized in 10 ml of antigen extraction buffer (Appendix VIII) and expressed through cheese cloth. 0.8 ml of the expressed sap was transferred into a 1.5 ml eppendorf tube and it was mixed with 0.4 ml of chloroform. The tube was vortexed and subjected to centrifugation at 12000 for 2 min. Later, 200 μl of clarified sap was transferred into 800 μl antigen extraction buffer and it was vortexed again. Nitrocellulose membrane (NCM) was cut into desired size, 3X1 cm and it was divided into lattices of 1X1 cm each with a soft lead pencil. The NCM was made wet by floating in tris- buffer saline (TBS) and was then air dried. On this, 10 μl of test

samples (diseased and healthy) were spotted. Then the NCM was air dried and immersed in blocking solution (5% SDM) with gentle oscillation for one hour, at room temperature. After this period, the NCM was rinsed once in TBS for 10 min. Then the membrane was incubated for one hour at room temperature, in crude antiserum diluted in TBS- SDM. The antibodies which gave positive indication in ELISA were used. Later it was rinsed thrice in TBS for 10 min each. Enzyme-linked anti- rabbit IgG was diluted in TBS- SDM, the membrane was incubated in it for one hour at room temperature and rinsed thrice in TBS for 10 min each. After washing and air drying, the membrane was incubated in substrate solution at room temperature in dark, and observed for development of purple colour after 15 min. The intensity of the colour was read using gel documentation system Gel DOC TM XR+).

3.5. Molecular diagnosis

The virus was subjected to molecular diagnosis after extracting the genetic material. Both DNA and RNA were extracted and subjected for PCR studies.

3.5.1. ***Isolation of genomic RNA***

1 ml of Trisol solution and 200 µl of β- mercaptoethanol were added to 100 mg of liquid nitrogen- ground leaf sample and centrifuged at 12,000 rpm for 10 min at 4°C. The clear supernatant was transferred to a new tube and incubated for five minutes at room temperature. Chloroform was added at the rate of 200 µl per ml of Trisol and the tube was shaken vigorously for 15 seconds. Tube was then incubated for 2-3 min at room temperature. After that the tubes were centrifuged at 12,000 rpm for 15 min at 4°C. The upper clear aqueous phase of the sample was pipette out for the isolation of RNA. 500 µl of 100% isopropanol was added to this clear phase, mixed gently and it was incubated for 10 min at room temperature. Then it was centrifuged at 12,000 rpm for 10 min at 4°C. After the centrifugation the supernatant was discarded and the RNA pellet was retained. The pellet was washed with 1 ml of 75% ethanol, vortexed briefly and centrifuged at 7500 rpm for 5 min at 4°C. After this the ethanol was discarded and the pellet

was air dried for 10 minutes. It was resuspended in 30 µl of RNAase- free water, incubated for 15 min in hot water bath of 55- 60°C temperature and stored at -80°C. This RNA was transcribed into cDNA form and PCR was carried out.

3.5.2. ***cDNA synthesis***

1. The following mixture was prepared in a PCR tube:

5 µl RNA (10 ng – 5µg total RNA containing 1 ng – 0.5 µg mRNA).

0.5 µl Primer: 50 µM Random hexamer.

2. The mixture was heated at 65°C for 5 min and cooled down immediately on ice. Then the tube was centrifuged briefly.

3. The reaction mixture was made with the following condition:

Pre-heated product (RNA& primer)

5X RT reaction buffer (incl. 10mM dNTP mix)- 2 µl

8 mM DTT- 0.5 µl

Rtase- 0.5 µl

RNase inhibitor- 10 µl

RNase- free water- to 20 µl

4. Mixed gently.

5. Incubated at 50°C for 60 min.

6. The reaction was inactivated by heating the reaction mixture at 95°C for five minutes.

3.5.3. ***Isolation of genomic DNA***

The genomic DNA was isolated using modified CTAB method (Lodhi *et al.*,1994) (Appendix VII) as well as by using DNeasy plant mini kit

(Qiagen). The protocol for genomic DNA isolation using modified CTAB method is given below:

1 .100 mg of fresh leaf tissue with distinct symptoms was taken in a sterile mortar, added liquid nitrogen to freeze the sample and powdered using a sterile pestle.

2. The powdered sample was mixed well with1ml of preheated 60°C extraction buffer.

3. Transferred 750 µl of sample to a microfuge tube and heated at 60°C for 30 minutes.

4. The samples were then centrifuged at 10000 rpm for 10 minutes.

5. To the supernatant added 10 µl of RNAase and incubated for 37°C for 1 hr.

6. This was mixed with equal volume (750 µl)of chloroform: isoamyl alcohol

(24:1 mixture) and centrifuged at 15000 rpm for 10 minutes.

7. The top aqueous layer is transferred to a microfuge tube and added 0.8 µl of ice cold isopropanol and incubated for 1hr at -20°C.

8. Centrifuged at 15000 rpm at 4°C for 10 minutes.

9. Discarded the supernatant and washed the pellet in 0.5 ml of 70% ethanol.

10. Centrifuged at 12000 rpm for 5 minutes.

11. Pellet was air dried to remove the ethanol.

12. It was suspended in 50µl of 1X TE buffer (AppendixIV) and stored at – 20°C.

3.5.3. ***Agarose gel electrophoresis***

Confirmation of the RNA as well as DNA was done by running the samples separately in 0.8 % agarose gel prepared in TAE buffer (Appendix X) with 1 µl of ethidium bromide (EtBr) added to the gel and casted in a horizontal gel electrophoresis unit (BioRad Power pack, U.S.A).100 bp ladder (GeNei) was

used as molecular marker and 2 µl of DNA was mixed with 1 µl of 6 X loading dye was dispensed into wells of the gel. The gel was run at $5Vcm^{-1}$ in TAE buffer. Gel was removed when the dye has run three fourth of the total distance and this was visualized in a UV transilluminator system (Bio-Rad) and documented in Gel Doc system(Gel DOC ™ XR+).

3.5.4. *PCR Amplification*

The cDNA and the isolated genomic DNA were subjected to PCR amplification with the following primers which are specific for *Banana bract mosaic virus, Cucumber mosaic virus* as well as a pair of degenerate primers each for begomoviruses and potyviruses.

3.5.4.1. Primers used

3.5.4.2. PCR Analysis

Virus	Direction	Sequence (5′→3′)	Reference
Begomoviruses	Forward	GCCHATRTAYAGRAAGCCMAGRAT	Wyatt and Brown, 1996.
	Reverse	GGRTTDGARGCATGHGTACANGCC	
Potyviruses	Forward	GTITGYGTIGAYGAYTTYAAYAA	Zheng *et al.*, 2008.
	Reverse	TCIACIACIGTIGAIGGYTGNCC	
BBrMV	Forward	TCTGGAACGGAGTCAACC	Zhang *et al.*, 2016.
	Reverse	CCGTGACATTACGCATTG	
BBrMV Flowering ginger	Forward	TCATTACAGCGAACCAGCAG	Designed
	Reverse	TCGTAGCGTGCTTTCATCAC	
CMV	Forward	ATGGACAAATCTGAATCAACC	Khan *et al.*, 2011
	Reverse	TCAAACTGGGAGCACCCC	

PCR amplification reactions were carried out in a 20 µl reaction volume which contained: 10X Taq Buffer, 1.5 mM $MgCl_2$, 2.0 mM each dNTPs (dATP, dGTP, dCTP and dTTP), 3 µl cDNA or genomic DNA, 0.2 µl Taq DNA polymerase, 1 µl each of forward and reverse primers and distilled water to 20 µl.

3.5.4.3. PCR amplification profile for the different primers used

PCR amplification profile for Begomovirus Primers AV494-F and AC1048-R.

95°C	-	1 min	
95°C	-	1 min	35 cycles
58°C	-	1 min	
72°C	-	1 min	
72°C	-	10 min	
4°C	-	∞	

PCR amplification profile for BBrMV Primers.

92°C	-	1 min	
92°C	-	55 sec	35 cycles
58.3°C	-	1 min	
72°C	-	50 sec	
72°C	-	10 min	
4°C	-	∞	

PCR amplification profile for Potyviruses Nib2-F and Nib3-R.

92°C	-	1 min	
92°C	-	55 sec	35 cycles
52°C	-	1 min	
72°C	-	50 sec	
72°C	-	10 min	
4°C	-	∞	

PCR amplification profile for BBrMV (Flowering ginger) Primers.

92°C	-	1 min	
92°C	-	55 sec	35 cycles
59°C	-	1 min	
72°C	-	50 sec	
72°C	-	10 min	
4°C	-	∞	

PCR amplification profile for CMV Primers.

95°C	-	5 min	
95°C	-	1 min	35 cycles
57.8°C	-	1 min	
72°C	-	1 min	
72°C	-	7 min	
4°C	-	∞	

3.5.4.4. Agarose gel electrophoresis of PCR products

The PCR products were checked in 1.2 per cent agarose gels prepared in 1X TAE buffer containing 1 per cent ethidium bromide (EtBr) (Appendix X). 2 µl of 6X loading dye was mixed with 15 µl of PCR product and loaded in the wells of the agarose gel. Electrophoresis was carried out for 1-2 hours. The molecular standard used was 100 bp DNA ladder (Origin). The gels were visualized in a UV transilluminator system (Bio-Rad).

3.6. Characterization

CP genes of the virus were sequenced after PCR. The PCR products along with the primers were submitted at Agri Genome Labs Private Limited, Smart City, Kochi for the sequencing of the coat protein genes (Appendix XI).

The gene sequence of the virus was analyzed using BLAST for finding out the sequence similarity with sequences available in Genebank. The sequences obtained in the blast output were aligned using Clustal-W software and the cluster dendrogram was constructed using PHYLIP package.

3.7. Management

A pot culture trial was conducted as part of the study for evaluating the effects of different antiviral principles like botanicals, chemicals and that of microbial origin against the virus infection in ginger. The study was conducted in completely randomized design (CRD) of 13 treatments with three replications each. The variety of the seed material used was Karthika and it was purchased from the Instructional Farm, College of Agriculture, Vellayani. The rhizomes were collected from diseased plants with 100% disease incidence.

Treatments:

T1: Perfekt 0.5 ml/L

T2: Perfekt 1.0 ml/L

T3: Aspirin 100 ppm

T4: Aspirin 150 ppm

T5: Salicylic acid 100 ppm

T6: Salicylic acid 150 ppm

T7: Barium chloride 100 ppm

T8: Barium chloride 150 ppm

T9: *Mirabilis jalapa* 10% leaf extract

T10: *Bougainvillea spectabilis* 10% leaf extract

T11: Neem oil – garlic emulsion 2%

T12: PGPR mix II -2%

T13: Untreated control.

The treatments were given at fortnightly interval. Before each spray the efficacy of the treatments were evaluated by calculating the vulnerability index (V.I) developed by Bos (1982) of the plants.

For calculating the vulnerability index, score chart of the disease was prepared with six scores ranging from zero to five, explained as follows (Plate 1):

0: Healthy leaves

1: Pale green leaves with small flecks

2: 25-50% mosaic and streaks on leaves

3: Streaks on 75% of the leaf

4: Necrotic streaks and veinal necrosis

5: Severe necrosis, stunting and drying

Scores: 0 1 2 3 4 5

Plate 1. Scoring of the disease according to symptoms

$$V.I = \frac{0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4 + 5n_5}{n_t(n_c - 1)}$$

n_x: number of plants in the respective category

n_t: total number of plants

n_c: total number of categories

The treatment which gave the least value of V.I was assessed as the best in management of the disease.

Results

4. RESULTS

In the present study, the symptomatology, transmission, biochemical analyses, immunological and molecular detection and management studies were carried out. Following results were obtained in the present investigation.

4.1. SYMPTOMATOLOGY

The symptoms of the disease appeared distinguishably on the young, newly emerging leaves of ginger plant. The symptoms first appeared on the leaves as small light green flecks (Plate 2). These flecks eventually increased in size and formed spindle shaped streaks (Plate 3). In certain plants the symptom expression initiated as appearance of light and dark green shades on the leaves, as mosaic symptom. However, this mosaic symptom was expressed only in the initial stage and the further emerged leaves showed spindle shaped streaks.

The streaks were centred on veins from mid rib and extended to the leaf margin. The streaks later turned to vein bulging on both sides. In most of the cases there were numerous spindle shaped streaks on a leaf. This imparted the leaves a mosaic appearance (Pate 4).

As the leaves matured, the streaks turned chlorotic spots. The appearance of too many streaks on the leaves led to severe chlorosis of the plant and they showed necrotic symptoms. These necrotic patches on the leaves imparted a dried appearance to the plants (Plate 5).

There was no visible symptoms on the leaf sheaths, pseudostem and rhizomes of the infected plants. The rhizomes collected from virus- infected plants as well as healthy plants did not vary in physical appearance.

4.2. TRANSMISSION

4.2.1. *Mechanical transmission*

The mechanical transmission of the virus was done on 50 young ginger seedlings with the virus inoculum homogenized in phosphate buffer (pH 7.0).

Plate 2. Appearance of chlorotic flecks in the leaves

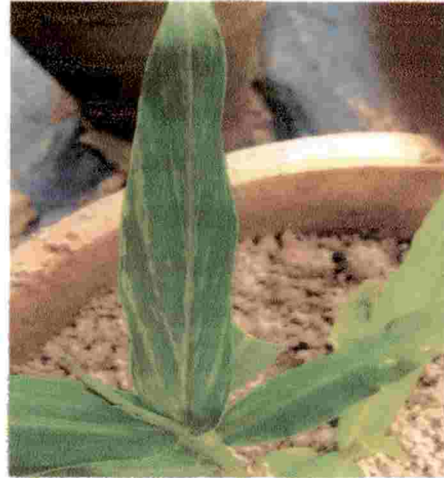


Plate 3. Slender spindle shaped streaks

Plate 4. Mosaic pattern on the leaves

Plate 6. Necrotic spots formation

After inoculation the seedlings were examined regularly for the appearance of mosaic symptom up to 120 days. None of the plants were found to develop symptoms which indicated that it was not mechanically transmitted.

4.2.2. *Transmission through planting material*

Rhizomes from both healthy and infected plants were used for the study. They were planted separately in pots. Small chlorotic flecks were observed in newly emerged leaves of infected rhizomes (Plate 6). The streaks increased in size periodically. The further emerged leaves also showed clear spindle shaped chlorotic streaks centred on the veins. These streaks were arranged parallel to each other from mid rib to the leaf margin. As the leaves matured the streaks also grew in size and the leaves started turning chlorotic. The streaks which were light green initially, became chlorotic by second month and became necrotic within a fortnight.

4.3. HOST – PATHOGEN INTERACTION

Changes in total carbohydrates, chlorophyll, phenol, total soluble proteins and defence related enzymes viz. peroxidase, polyphenol oxidase and phenylalanine ammonialyase were carried out at 30, 60, 90 and 120 days after the viral infection. Healthy plants without infection were also maintained as control. The protein profile and isozyme analysis of healthy and inoculated plants were also conducted.

4.3.1. Estimation of total carbohydrates

The total carbohydrate content in healthy and inoculated plants was estimated and is presented in Table 1. The results indicated that healthy plants showed more carbohydrate content with respect to the infected plants. In both healthy as well as infected plants, the carbohydrate level was observed to be increasing up to 60 and 90 DAI, after which a decline was observed. The healthy plants exhibited the highest carbohydrate level at 60 DAI and it was observed to be 23.61 mg g^{-1}. The infected plants showed the highest carbohydrate level at 90

Plate 6. Appearance of symptoms in rhizome transmission

DAI and it was 14.72 mg g^{-1}, and thereafter declined to 11.92 mg g^{-1} and 7.26 mg g^{-1} respectively.

Table 1. Changes in total carbohydrate content of ginger leaves due to viral infection, mg g^{-1}.

Days after infection	Change in carbohydrate content (mg g^{-1} on fresh weight basis)*		Per cent decrease over healthy
	Healthy	Infected	
30	14.42	8.62	40.22
60	23.61	11.39	51.76
90	16.08	14.72	8.46
120	11.92	7.26	39.09

CD at 5%: Healthy X Infected: 1.50

DAI: 1.06

* Mean of three replications

4.3.2. Estimation of chlorophyll

The estimation of chlorophyll in healthy and infected plants comprised of estimations of chlorophyll a, chlorophyll b and total chlorophyll content at different days of interval. Throughout the estimation the chlorophyll content was found much lower in infected plants when compared to healthy plants. The healthy plants showed an increase in total chlorophyll content, from 1.73 mg g^{-1} at 30 DAI to 1.97 mg g^{-1} at 120 DAI, whereas infected plants showed a decline from 1.14 mg g^{-1} to 0.56 mg g^{-1} during the same period of observation. The chlorophyll a content of infected plants declined to the level of 0.37 mg g^{-1} at 120 DAT from 0.74 mg g^{-1} at 30 DAI. During the same period the healthy plants had an increase in chlorophyll a content from 1.25 mg g^{-1} to 1.44 mg g^{-1}. Similarly in healthy

plants, the chlorophyll b content increased from 0.49 mg g^{-1} at 30 DAI to 0.53 mg g^{-1} at 120 DAI. But the chlorophyll b content of infected plants showed a reduction of 0.19 mg g^{-1} at 120 DAI from 0.40 mg g^{-1} at 30 DAI. The chlorophyll a, chlorophyll b and total chlorophyll contents showed its peak at 60 DAI, after which response was erratic (Table 2).

The results revealed that the infected plants showed a significant reduction in the chlorophyll content than in healthy plants due to the viral infection.

Table 2. Changes in total chlorophyll content of ginger leaves in response to virus infection, mg g^{-1}.

Days after infection	Changes in chlorophyll content (mg g^{-1} on fresh weight basis)*					
	a		b		Total	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
30	1.25	0.74	0.49	0.40	1.73	1.14
60	1.76	0.90	0.82	0.44	2.58	1.34
90	1.28	0.40	0.51	0.21	1.79	0.61
120	1.44	0.37	0.53	0.19	1.97	0.56

* Mean of three replications

CD at 5%	Chl a	Chl b	Total
Healthy Vs. Infected	0.077	0.023	0.091
DAI	0.109	0.032	0.128

4.3.3. Estimation of phenol

Phenolics and their oxidation products are the chemical substances produced in plant tissues in response to injury or infections. Study on the accumulation of phenolics in ginger leaves was undertaken and the estimation was done in both infected and healthy plants at different days of interval. The phenol

content was found to be more in the infected plants and was found to be increasing in the subsequent stages of infection. At 30 DAI, the infected plants showed 3.09 mg g^{-1} of phenol content while healthy showed 2.21 mg g^{-1}. The phenol content in infected and healthy plants at 120 DAI was estimated to be 8.30 mg g^{-1} and 3.56 mg g^{-1} respectively. Throughout the period of study, the phenol content in both infected and healthy ginger plants was showing an increasing trend except at 120 DAI in healthy plants and 90 DAI in infected plants, where the plants showed a slight decrease to 3.56 mg g^{-1} and 6.22 mg g^{-1}, respectively (Table 3).

Table 3. Changes in phenol content of ginger due to viral infection, mg g^{-1}

Days after infection	Change in phenol content (mg g^{-1} on fresh weight basis)*		Per cent increase over healthy
	Healthy	Infected	
30	2.21	3.09	39.82
60	3.78	6.48	71.43
90	5.42	6.22	14.76
120	3.56	8.30	133.15

* Mean of three replications

CD at 5%: Healthy Vs. Infected : 0.029

DAI : 0.041

4.3.4. Estimation of total soluble protein

The total soluble protein content was estimated in virus inoculated and healthy ginger plants as per the procedure described by Bradford (1976). The results showed lower protein content in infected plants in comparison with the healthy plants. The healthy plants showed an increasing trend and the infected plants showed a decreasing trend in the total soluble protein content. In healthy

plants the protein content was recorded 229.00 mg g^{-1} at 30 DAI and increased to a maximum of 264.67 mg g^{-1} at 120 DAI. In infected plants the maximum protein content recorded was 165.33 mg g^{-1} at 30 DAI and it decreased to 114.33 mg g^{-1} at 120 DAI. The results of protein estimations are represented in the Table 4. The results revealed that the infected plants showed a significant reduction in the protein content than in healthy plants due to the viral infection.

Table 4. Changes in total soluble protein content of ginger leaves in response to viral infection, mg g^{-1}

Days after infection	Change in soluble protein content (mg g^{-1} on fresh weight basis)*		Per cent decrease over healthy
	Healthy	Infected	
30	229.00	165.33	27.8
60	239.33	124.00	48.19
90	256.50	115.00	55.17
120	264.67	114.33	56.80

* Mean of three replications

CD at 5%, Healthy Vs. Infected : 3.42

DAI : 12.58

4.3.5. Estimation of defence related enzymes

4.3.5.1. *Estimation of peroxidase (PO)*

Peroxidase activity in healthy and inoculated ginger plants at different DAI was estimated (Table 5). A significant increase in the PO activity was noted in infected plants at all stages of observation, except at 30 DAI where PO activity in both healthy and diseased plants were statistically on par. The activity of the enzyme was found highest (54.50 min^{-1} g^{-1}) at 90 DAI whereas in healthy plants it recorded a lower value of 40.80 min^{-1} g^{-1}. A significant increase in PO activity

was noted at 60 DAI (51.43 min^{-1} g^{-1}) in infected plants which was found statistically on par with the PO activity at 90 and 120 DAI, recorded as 54.50 min^{-1} g^{-1} and 41.87 min^{-1} g^{-1} respectively. In healthy plants PO activity of all stages differed significantly, except at 30 and 60 DAI.

Table 5. Changes in peroxidase activity in ginger leaves in response to viral infection

Days after infection	Peroxidase activity (changes in absorbance min^{-1} g^{-1} on fresh weight basis)*		Per cent increase over healthy
	Healthy	Infected	
30	13.37	21.33	59.54
60	26.97	51.93	92.55
90	40.80	54.50	33.58
120	21.83	47.87	119.29

* Mean of three replications

CD at 5%: Healthy Vs. Infected: 10.14

DAI : 14.33

4.3.5.2. *Estimation of polyphenol oxidase (PPO)*

PPO activity in healthy and inoculated ginger at different DAI was estimated. The activity of PPO at 30 DAI in healthy (0.50 min^{-1} g^{-1}) was found on par with that of infected plants (0.93 min^{-1} g^{-1}). There was a significant difference between PPO activity of healthy and infected plants. At each stage of estimation both healthy and infected plants recorded significantly different values. The highest PPO activity was recorded in infected plants at 90 DAI and it was 13.43 min^{-1} g^{-1}. There was a decline in PPO activity in healthy as well as infected plants at 120 DAI, which was recorded to be 1.77 min^{-1} g^{-1} and 3.07 min^{-1} g^{-1} respectively in healthy and infected plants (Table 6).

Table 6. Changes in polyphenol oxidase activity in ginger due to viral infection

Days after infection	Polyphenol oxidase activity (changes in absorbance min^{-1} g^{-1} on fresh weight basis)*		Per cent increase over healthy
	Healthy	Inoculated	
30	0.50	0.93	86.67
60	1.43	2.20	53.85
90	5.40	13.43	148.77
120	1.77	3.07	73.45

* Mean of three replications

CD at 5%: Healthy Vs. Infected: 0.42

DAI : 0.601

4.3.5.3. *Estimation of phenylalanine ammonia-lyase (PAL)*

From the estimation of PAL activity in healthy and infected plants, it was observed that the enzyme activity was higher in inoculated plants except at 30 DAI, where it was found on par. Up to 90 DAI, the enzyme activity was found to increase and then to decline at the next stage of observation, in both healthy and infected plants. At all stages of observation the healthy plants showed significant difference in PAL activity while the infected plants showed significant increase to 120.28 µg g^{-1} min^{-1} at 90 DAI, after which a slight decline to 117.36 µg g^{-1} min^{-1}, which is statistically on par.

Table 7. Changes in phenylalanine ammonia-lyase activity in ginger due to viral infection

Days after infection	Phenylalanine ammonia-lyase activity (changes in absorbance µg g^{-1} min^{-1} on fresh weight basis)*		Per cent increase over healthy
	Healthy	Infected	
30	95.83	90.56	5.50
60	75.97	95.28	25.42
90	96.11	120.28	25.15
120	40.83	117.36	187.44

* Mean of three replications

CD at 5%: Healthy Vs. Infected: 7.29

DAI : 10.30

4.3.6. Electrophoretic analysis of proteins using SDS – PAGE

Protein profile of healthy and infected ginger samples were analyzed using SDS PAGE. An additional band in their protein profile of the virus infected samples, which indicated the presence of a novel protein with molecular weight of 20 kDa (Plate 7).

4.3.7. Isozyme analysis of enzyme

Native polyacrylamide gel electrophoresis was carried out for isozyme analysis of peroxidase to find out the variation in the isozyme content of healthy and diseased plants. The relative mobility (Rm) of each band was calculated. The peroxidase activity in the virus infected samples showed the presence of definite single band with Rm value of 0.812 (Plate 8).

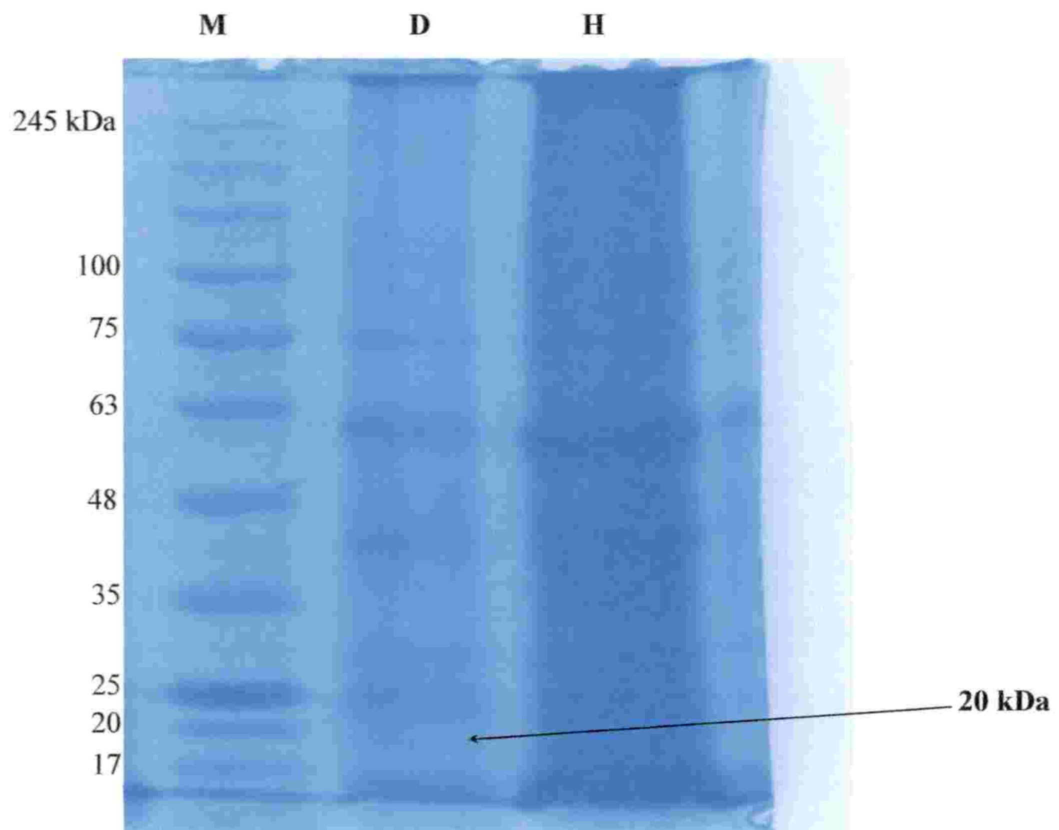
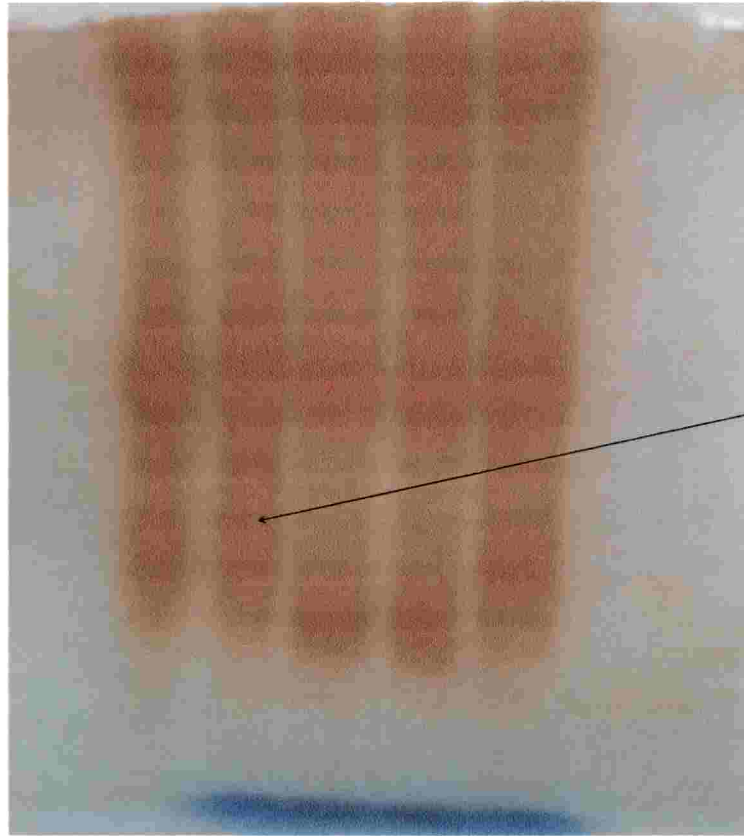


Plate 7. Protein profile analysis using SDS PAGE

H – Healthy
D – Diseased
M - Marker



R_m: 0.812

D D H H D

Plate 8. Peroxidase isozyme profile in healthy and diseased samples

H – Healthy
D – Diseased

174317

4.4. Immunological and molecular detection

The immunological detection techniques namely direct antigen coating-enzyme linked immunosorbant assay (DAC- ELISA), triple antibody sandwich ELISA (TAS- ELISA) and dot immunobinding assay (DIBA) were carried out.

4.4.1. DAC- ELISA

DAC- ELISA was done for different infected leaves samples collected from field. Polyclonal antibody (Agdia Pvt Ltd) was used for detecting the presence of *Banana bract mosaic virus* (BBrMV), *Cucumber mosaic virus* (CMV), *Sugarcane bacilliform virus* (SCBV), *Southern bean mosaic virus* (SBMV) and *Tobacco mosaic virus* (TMV). The absorbance was measured at 405 nm in an ELISA reader (BIORAD microplate reader). The results of the experiment revealed that among the antibodies tested, BBrMV and CMV showed positive result while the others gave negative result (Table 8).

Table 8. Reaction of ginger samples infected by virus in DAC- ELISA.

Sl. No.	Antibody	Absorbance at 405 nm*		Remarks
		Healthy	Diseased	
1	BBrMV	0.05	0.15	Positive
2	CMV	0.07	0.19	Positive
3	SCBV	0.253	0.233	Negative
4	SBMV	0.351	0.329	Negative
5	TMV	0.081	0.079	Negative

* Mean of three replications

4.4.2. TAS- ELISA

TAS- ELISA was carried out using the polyclonal antibodies for *African cassava mosaic virus* (ACMV) and *Sri Lankan cassava mosaic virus* (SLCMV) (DSMZ, Germany). The absorbance was measured at 405 nm in BIORAD

microplate reader. The absorbance value of ACMV indicated positive result (Table 9).

Table 9. Reaction of ginger samples infected by virus in TAS- ELISA.

Sl. No.	Antibody	Absorbance at 405 nm*		Remarks
		Healthy	Diseased	
1	ACMV	0.017	0.0425	Positive
2	SLCMV	0.122	0.150	Negative

* Mean of three replications

4.4.3. Dot immunobinding assay (DIBA)

DIBA was conducted to detect the presence of BBrMV and CMV based on the results of ELISA. Polyclonal antibodies for BBrMV and CMV (Agdia Pvt Ltd) were used for the study. The result of the experiment was analyzed by comparing development of purple colour in a gel documentation system (BIORAD Gel Doc^{TM} XR+). Reaction of infected samples with polyclonal antibody of BBrMV showed purple coloured spots in the nitrocellulose membrane (NCM) (Plate 9). The reaction was negative with antibody specific to CMV.

4.5. Molecular diagnosis

Molecular diagnosis using Polymerse chain reaction (PCR) was performed for the detection of Begomoviruses, where RT- PCR was performed for the detection of BBrMV and CMV genomes. RNA was isolated using Trisol method (Plate 10) and DNA was isolated using CTAB method (Plate 11).

A pair of degenerate primers for begomovirus genus and a pair for Potyviridae family were used for the detection of the suspected viruses. Primer pair specific for coat protein gene was used for the detection of BBrMV, CMV and BBrMV isolate from flowering ginger (*Alpinia purpurata*).

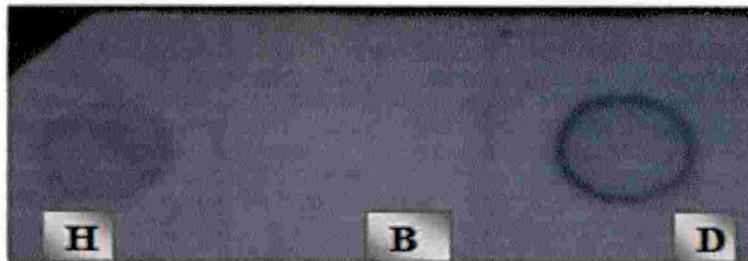


Plate 9 a. Reaction of CMV in DIBA

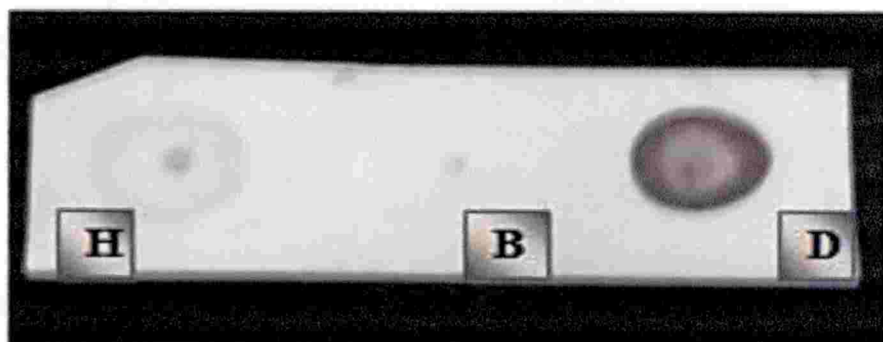


Plate 9 b. Reaction of BBrMV in DIBA

H: Healthy
B: Blank
D: Diseased

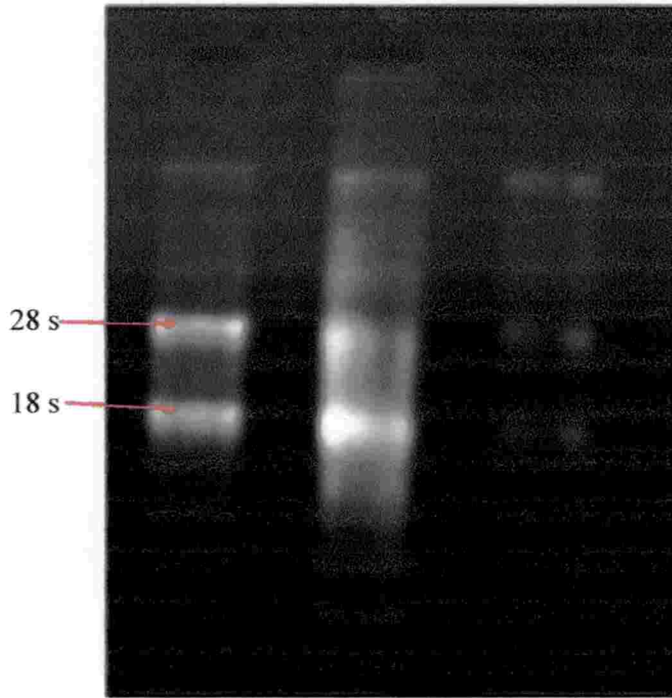


Plate 10. RNA isolated

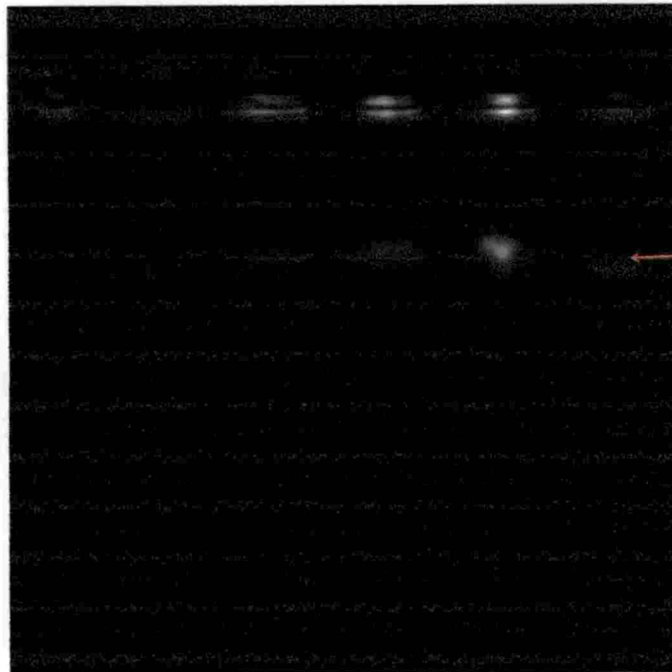


Plate 11. DNA isolated

PCR was carried out for 35 cycles and the product was subjected to electrophoresis in an electrophoresis unit (BIORAD Mini-Sub® Cell GT). A molecular weight marker (Origin 100 bp DNA ladder) was also loaded along with the PCR product. An amplicon of size 550 bp (Plate 12) was obtained in begomovirus degenerate primer (AV494- F and AC1048- R). None of the other primers used for the suspected viruses gave any amplifications in the PCR.

4.6. Sequencing of the PCR product

The PCR product of begomovirus isolate (Appendix) was sequenced at AgriGenome Labs private limited, Smart City, Kochi. The sequence was analyzed using BLAST software (Plate 13). 17 related sequences were selected (Table 10) and cluster dendrogram was constructed using CLUSTAL W software (Plate 14). The BLAST analysis indicated 74% similarity to *Tomato leaf curl virus Bangalore isolate*.

4.7. Management

The management studies were conducted with antiviral principles like botanicals, chemicals and that of microbial origin against the virus. The experiment was conducted in completely randomized design (CRD) with 13 treatments and three replications. Karthika was the variety used for the study. Vulnerability index (V.I.) was calculated after application of treatments, as described by Bos (1982). Three sets of sprays were given at fortnightly interval. V.I. before first, second and third sprays and two weeks after third spray were calculated and the values are expressed in Table 11.

The V.I. values calculated before first spray were ranging from 35.66 to 48.67, which were not significantly different. After the first spray, V.I. values were calculated just before the second spray. It was observed that the least V.I. was estimated from the plants treated with Perfekt at 1.0 ml L^{-1}(V.I. 37.09) and 10% leaf extract of *Mirabilis jalapa* (V.I. 37.03). These treatments were found statistically on par and superior to other treatments.

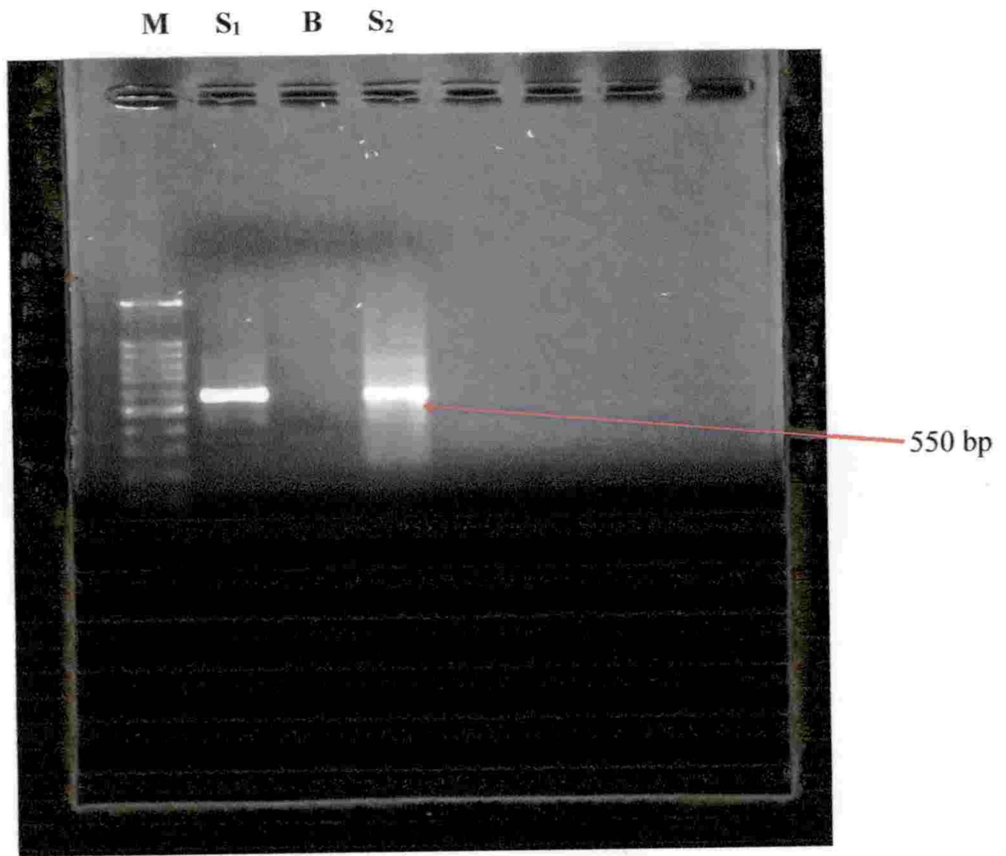


Plate 12. PCR amplification for Begomoviruses

M: 100 bp DNA ladder
S_1: Sample 1
B: Blank
S_2: Sample 2

BLAST Results

Edit and Resubmit Save Search Strategies ▷Formatting options ▷Download How to read this page Blast report description

Job title: Ginger.begomo_AV.forward_6874-1_P0941,Raw...

RID GCZ8GJYW014 (Expires on 05-25 16:52 pm)

Query ID lcl|Query_8891
Description Ginger.begomo_AV.forward_6874-1_P0941,Raw Sequence(602 bp)
Molecule type nucleic acid
Query Length 602

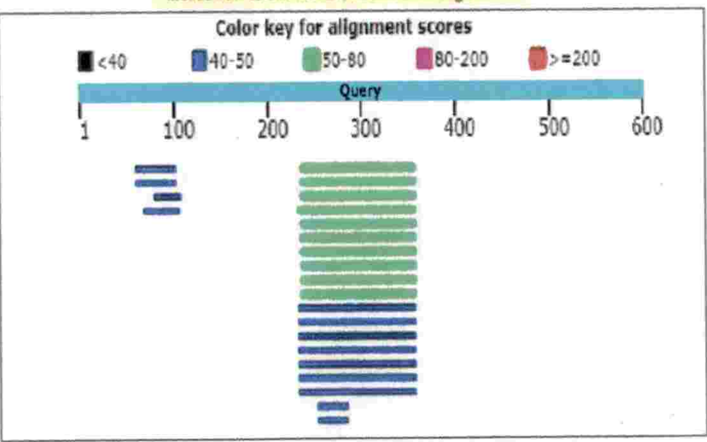
Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.8.0+ ▷Citation

Other reports: ▷Search Summary [Taxonomy reports] [Distance tree of results] [MSA viewer]

Graphic Summary

Distribution of the top 23 Blast Hits on 23 subject sequences

Mouse over to see the title, click to show alignments



Activate Windows

Plate 13. BLAST results obtained for the sequence

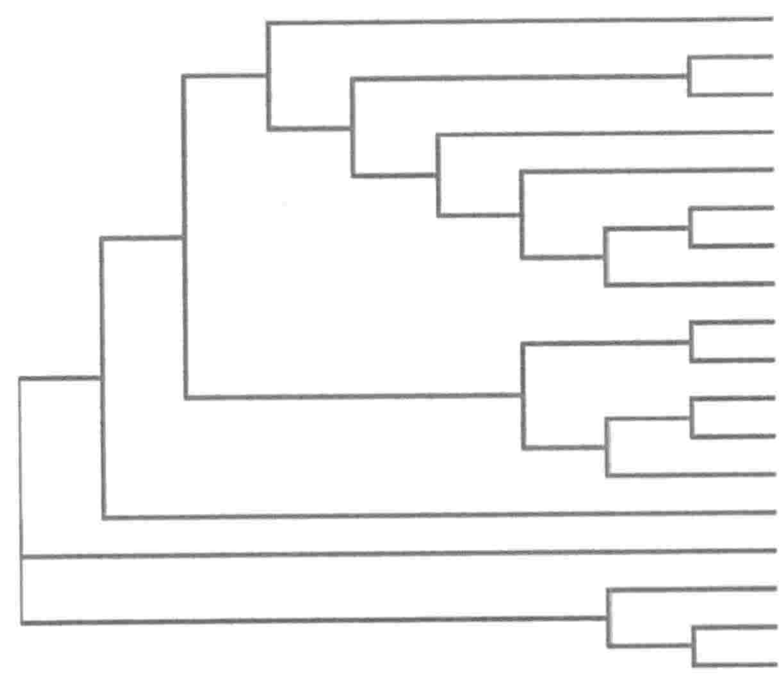


Plate 14. Cluster dendrogram showing the relationship of begomovirus isolate with other related sequences

The V.I.. values estimated before third spray revealed that Perfekt at 0.5 ml L^{-1} (V.I. 37.90), Perfekt at 1.0 ml L^{-1}(V.I. 37.07) and 10% *M. jalapa* (V.I. 37.10) were superior than the other treatments. After two weeks of the third spray V.I. values were calculated. Among the values, the least values were 30.66 and 31.42, from the plants which were treated with Perfekt at 0.5 ml L^{-1} and 1.0 ml L^{-1}, respectively. The second best treatment was observed to be 10% leaf extract of *M. jalapa* with a V.I. value of 38.15.

The antiviral chemicals used in this study were Aspirin, Salicylic acid and Barium chloride at two concentrations, 100 ppm and 150 ppm. Aspirin treatment to the plants resulted in reducing the V.I. values to 47.05 and 45.25 at 100 ppm and 150 ppm respectively, as compared to 79.15, which was the V.I. value of untreated control after the first spray. Similarly, after the second spray, the untreated plants expressed V.I. value of 80.35, as against 48.46 and 48.44 in the aspirin treatment at 100 ppm and 150 ppm respectively. After two weeks of the third spray, V.I. value of untreated plants were 100, where that of aspirin treated plants were 52.03 at 100 ppm and 55.55 at 150 ppm. Aspirin treatment at these two concentrations brought an average symptom inhibition of 46.21% over the untreated plants.

Salicylic acid (SA) was used at 100 and 150 ppm concentrations. After the first spray, the treated plants expressed a V.I. value of 48.73 and 51.01 at 100 ppm and 150 ppm respectively, while the untreated plants showed a value of 79.15. After the second spray, control plants were showing a V.I. value of 80.35, while the SA treated plants showed 48.44 at 100 ppm and 50.74 at 150 ppm of SA treatment. The third spray of SA reduced the V.I. of treated plants to 56.44 and 63.54 at 100 and 150 ppm respectively, whereas the control plants recorded with a V.I. of 100. SA treatment resulted in 40.01% suppression of symptoms over the untreated control.

Barium chloride treatment at 100 ppm and 150 ppm was found to give V.I. values of 49.80 and 50.59 respectively after first spray, 50.56 and 52.28

respectively after second spray and 61.42 and 64.83 after the third spray. The V.I. values of untreated plants were 79.15, 80.35 and 100, respectively which were significantly higher than that of treated plants.

The treatment using 10% leaf extract of *Bougainvillea spectabilis* gave the V.I. of the treated plants, 49.69, 41.94 and 45.28 after first, second and third sprays respectively. At each stage the V.I. value of untreated plants were 79.15, 80.35 and 100 respectively. In this treatment, it was found to be the third best treatment in disease suppression. After the third spray there was 54.72% suppression of infection over the control plants.

Neem oil- garlic emulsion (2%) and PGPR mix- II (2%) resulted in V.I. values of the treated plants which were not significantly different after the third spray. These two treatments resulted in an average suppression of 24.16% after the third spray, where the control plants were showing a V.I. of 100. These two treatments resulted in a very low suppression which not effective.

Table 10. Description of selected isolate for the construction of Cluster dendrogram.

Sl. No	Accession Number	Description	Similarity	E value
1	AY428770.1	Tomato leaf curl Bangalore virus isolate ToLCBV-AVT1 segment DNA A, complete sequence	74%	5e-05
2	KP164856.1	Tomato leaf curl Bangalore virus isolate TC217 segment DNA-A, complete sequence	73%	6e-04
3	KP164855.1	Tomato leaf curl Bangalore virus isolate TC220 segment DNA-A, complete sequence	73%	6e-04
4	AJ810369.1	Tomato leaf curl virus AV1 gene for coat protein , isolate 30	72%	6e-04
5	AF428255.1	Tomato leaf curl Bangalore virus-[Kolar] DNA A, complete genome	73%	6e-04
6	AY456684.1	Tomato leaf curl Bangalore virus-Cotton [Fatehabad] segment A, complete sequence	73%	6e-04
7	AF295401.1	Tomato leaf curl Bangalore virus-[Ban5] DNA A, complete genome	73%	6e-04
8	GU170806.1	Tomato leaf curl virus isolate ToLCV-To-KLR.1 segment DNA A, complete sequence	72%	0.026
9	GU474418.1	Tomato leaf curl Bangalore virus isolate 18 segment DNA-A, complete sequence	72%	0.026
10	AF165098.1	Tomato leaf curl geminivirus DNA-A strain LCV-Ban4, complete genome	72%	0.026
11	KP164857.1	Tomato leaf curl Bangalore virus isolate TC177 segment DNA-A, complete sequence	71%	0.32
12	KC222952.1	Tomato leaf curl virus isolate Hyderabad coat protein gene, complete cds	71%	0.32

13	DQ358098.1	Tomato leaf curl virus strain TNAU1 pre-coat protein (AV2) and coat protein (AV1) genes, complete cds	71	0.32
14	AJ810355.1	Tomato leaf curl virus AV1 gene for coat protein, isolate 16	71%	0.32
15	AJ810349.1	Tomato leaf curl virus AV1 gene for coat protein, isolate 10	71%	0.32
16	AY738102.1	Tomato leaf curl Bangalore virus from India coat protein (AV1) gene, partial cds; replication associated protein (AC3) and transactivator protein (AC2) genes, complete cds; and replicase (AC1) gene, partial cds	71%	0.32
17	Z48182.1	Tomato leaf curl virus - Bangalore I V1, V2, C1, C2, C3 and C4 genes	71%	0.32

Table 11. Vulnerability index (VI) of plants after the management study

Treatments	Vulnerability index*			
	Before first spray	Before second spray	Before third spray	Two weeks after third spray
T1 Perfekt 0.5 ml/L	41.07 [a]	40.90 [cd]	37.90 [f]	30.66 [h]
T2 Perfekt 1.0 ml/L	48.67 [a]	37.09 [d]	37.07 [f]	31.42 [h]
T3 Aspirin 100 ppm	45.96 [a]	47.05 [bgd]	46.78 [de]	52.03 [e]
T4 Aspirin 150 ppm	44.11 [a]	45.25 [cd]	48.46 [de]	55.55 [e]
T5 Salicylic acid 100 ppm	40.00 [a]	48.73 [bc]	48.44 [de]	56.44 [de]
T6 Salicylic acid 150 ppm	41.75 [a]	51.01 [bc]	50.74 [cd]	63.54 [c]
T7 Barium chloride 100 pp m	42.48 [a]	49.80 [bc]	50.56 [cd]	61.42 [cd]
T9 *Mirabilis jalapa* 10% le af extract	38.84 [a]	37.13 [d]	37.10 [f]	38.15 [g]
T10 *Bougainvillea spectab ilis* 10% leaf extract	41.75 [a]	49.69 [bc]	41.94 [ef]	45.28 [f]
T11 Neem oil–garlic emulsion 2%	48.08 [a]	56.92 [b]	58.84 [b]	77.76 [b]
T12 PGPR mix II -2%	48.40 [a]	56.85 [b]	58.40 [c]	73.93 [b]
T13 Untreated control.	47.39 [a]	79.15 [a]	80.35 [a]	100 [a]
CD (0.05)	15.199	10.209	7.852	5.247

*Mean of three replications

Discussion

5. DISCUSSION

Viral diseases have a huge impact on production, productivity and quality of a crop. Due to the lack of effective management practices, the viral diseases often destroy the crop. It is known that there are no chemical management strategies for viral diseases. But there is an emergence of many antiviral chemicals and antiviral principles which are plant based as well as of microbial origin. All of these are in use for reducing the disease incidence and withstanding the viral infection. Majority of these are having the activity of inducing resistance mechanisms in plants against viruses.

Ginger is an important spice crop in Kerala and a new viral mosaic infection is being noticed in the crop. This mosaic disease in ginger is an emerging field problem among farmers in the state. Hence this investigation was undertaken to study the symptomatology, transmission, host- pathogen interaction, immunological and molecular detection, characterization of the virus and management of the disease using antiviral principles of chemical, botanical as well as microbial origin. Discussion of the results obtained is presented in this chapter.

5.1. Symptomatology

The symptoms observed in this disease are in line with those reported by Nambiar and Sarma (1974). They observed appearance of chlorotic flecks in ginger leaves. They added that those flecks later developed into spindle shaped streaks. Similar symptomatology was reported by So (1980) from ginger plantations in Korea. He reported yellow and dark green mosaic symptoms in the plants and stunting of the plants which exhibited the mosaic symptoms. The occurrence of thin, slender, spindle shaped chlorotic flecks were reported by Thomas (1986) in ginger plants from Australia. He named it as *Ginger chlorotic fleck virus* (GCFV). He also noticed that the symptoms were first developed in younger leaves. He also stated that there were no visible symptoms on the rhizomes and pseudostem.

Similar symptomatology was reported in large cardamom (*Amomum subulatum* Roxb.), cardamom (*Elettaria cardamomum)* and flowering ginger (*Alpinia purpurata*) by Rayachaudhuri and Ganguli (1965), Siljo *et al.*(2012) and Zhang *et al.* (2016) respectively.

Rayachaudhuri and Ganguli (1965) reported mosaic symptoms on leaves of large cardamom. The mosaic was later observed to be coalescing and it resulted in drying up of leaves. Similar symptoms were reported from cardamom by Siljo *et al.*(2012). They diagnosed the disease with continuous or discontinuous chlorotic streaks along the veins and midrib. They reported it as a novel kind of viral infection symptom in cardamom and termed it as 'Chlorotic streak' in cardamom. Similar kind of mosaic and streak symptoms were observed in flowering ginger plants of Hawaii and was reported by Zhang *et al.* (2016). The symptoms described elsewhere were similar to that described in the present investigation.

5.2. Transmission

Transmission of the virus was tested in rhizome (seed material collected from infected plants) and mechanical inoculation using infected leaf sap to healthy plants. Small chlorotic flecks were observed to be appearing on the plants emerged from infected rhizomes. These streaks were found increasing in size periodically. The further emerged leaves showed clear spindle shaped streaks centred on veins. The virus could be transmitted through rhizome but not through mechanical transmission.

Transmission of the virus was studied by Nambiar and Sarma (1974) and they have reported that the mosaic disease of ginger could effectively be transmitted through infected rhizomes. They observed clear mosaic symptoms on newly emerging leaves of rhizomes which were collected from the virus- infected mother plants. They also failed in transmitting the virus mechanically to ginger plants and other test hosts such as *Nicotiana tabacum* var. *xanthi*, *N. tabacum* var. *rustica*, *N. tabacum* var. *harison special*, *N. glutinosa*, *Elettaria cardamomum*, *Curcuma longa* and *C. aromatica*.

So (1980) also studied the artificial inoculation by sap transmission of the mosaic disease of ginger. He stated that the virus was mechanically transmissible to 23 other species of plants, which were known to be susceptible to *Cucumber mosaic virus* (CMV). But low efficiency mechanical transmission of *Ginger chlorotic fleck virus* (GCFV) was observed by Thomas (1986). He also could not transmit the virus to any diagnostic hosts. Fan *et al.* (1999) stated that a virus isolate from ginger was mechanically transmissible to 25 plant species from five families.

Zhang *et al.* (2016) studied mechanical transmission of BBrMV from flowering ginger (*Alpinia purpurata*) to the leaves of *Cassia occidentalis, Chenopodium amaranticolor, Brassica campestris, Capsella rubella, Solanum lycopersicanum, Cucumis sativus, Vigna unguiculata, Phaseolus vulgaris* and *Alpinia purpurata.* They could not succeed in the transmission of the virus mechanically. They also couldn't transmit BBrMV mechanically to edible ginger.

5.3. Host – Pathogen interaction

Changes in total carbohydrates, chlorophyll, phenol, total soluble proteins and defence related enzymes viz. peroxidase, polyphenol oxidase and phenylalanine ammonialyase were carried out at 30, 60, 90 and 120 days after the viral infection. Healthy plants without infection were also maintained as controls. The protein profile and isozyme analysis of healthy and inoculated plants were also conducted.

The total carbohydrate content was found significantly higher in healthy plants than that of infected plants at all stages of infection. The results are in line with the findings of Ramiah (1978) who reported that there was decrease in synthesis of total carbohydrates in infected leaves of susceptible cowpea. A decrease of total sugar and starch in leaf tissues of sunhemp infected with *Bean mosaic virus* was reported by Singh and Singh (1980). The observation of Singh and Singh (1984) was the virus infection decreased the total sugar and starch in cowpea cultivars infected with *Southern bean mosaic virus* and *Cowpea mosaic virus*. Johri and Pandhi (1985) also reported similarly that the carbohydrate level declined positively with severity of disease symptoms in case of yellow vein mosaic of okra. Similarly BYVMV infected

okra plants were reported with low reducing sugars and increase in total sugar and non-reducing sugar contents by Sarma *et al.* (1995). Thind *et al.* (1996) reported that the amount of total sugars and starch decreased in black gram infected with *Yellow mosaic virus* when compared with healthy plants. Sutha *et al.* (1998 b) reported that TSWV infection reduced the concentration of total, reducing and non-reducing sugars of tomato fruits. Mali *et al.* (2000) reported that *Mungbean yellow mosaic virus* (MYMV) infection on moth bean resulted in significant reduction of total soluble carbohydrates in susceptible cultivar when compared to resistant cultivar. Sindhu (2001) also reported a reduction in the level of carbohydrate content in susceptible varieties inoculated with BlCMV compared to healthy control. Post infection decrease in reducing sugars, non-reducing sugars and total sugars in the plants infected with TLCV was reported by Raghavendra (2002). A significant reduction in total sugar content in BlCMV infected cowpea plants was observed by Krishnapriya (2015). Findings of the present study are in agreement with previous reports of decreased starch content in virus infected plants. This might be due to the alterations in photosynthesis and as a result of disruption of normal phloem transport (Estelitta *et al.*, 1993 and Bhagat and Yadav 1997). All these findings are in accordance with the present investigation.

The estimation of chlorophyll in healthy and infected plants comprised of estimations of chlorophyll a, chlorophyll b and total chlorophyll content at different days of interval. Throughout the estimation the chlorophyll content was found much lower in infected plants than healthy plants. Chlorophyll degradation is a common characteristic phenomenon in viral infections and the present findings are in terms with that of other reports. Alagianagalingam and Ramakrishnan (1979) observed a reduction in chlorophyll content in cassava leaves infected by the virus as compared to healthy leaves. Ayanru and Sharma (1982) also reported the similar reduction of chlorophyll a and b in mosaic virus infected cassava plants. The reduction in chlorophyll content of okra leaves due to BYVMV infection was reported by Sarma *et al.* (1995). Similarly a decline in chlorophyll content of mung bean plants infected

with yellow mosaic virus was observed by Thind *et al.* (1996). Vasanthi and Shanmugam (2003) observed lowest content of chlorophyll a, b and total chlorophyll in ICMV infected cassava as compared to healthy ones. Total chlorophyll, chlorophyll a and chlorophyll b were found to be lower in mung bean plants infected by *Mungbean yellow mosaic virus* (MYMV) (Momol and Pernezny, 2006). Arpita and Subrata (2008) observed a gradual fall in green pigments like chlorophyll a, b and total chlorophyll contents in mesta at different stages of yellow mosaic virus infection and there was alteration of the ratio between chlorophyll a and b which affects the photosynthetic efficiency. Philip (2010) reported a reduction in chlorophyll a and b in Cassava mosaic geminiviruses (CMG) infected cassava leaves compared to healthy leaves. Chlorophyll pigments a and b as well as total chlorophyll were found lower in finger millet plants due to mottle streak virus infection. From the studies of Ramiah (1978) and Ahmed *et al.* (1986) the possible reason for reduction of chlorophyll content in virus infected plants is the increased activity of chlorophyllase enzyme in them.

Study on the accumulation of phenolics in ginger leaves was undertaken and the estimation was done in both infected and healthy plants at different days of interval. The phenol content was found to be more in the infected plants and was found to be increasing in the subsequent stages of infection. Similar results were reported by Sharma *et al.* (1984) from their studies on effects of viral and fungal infections in musk melon and showed an increasing trend of enzyme activity and phenol component as compared to healthy control, irrespective of the nature of infection. Sohal and Bajaj (1993) reported an increase in total phenols in both resistant and susceptible varieties of black gram infected with *Yellow mosaic virus*. Sarma *et al.* (1995) reported increased total phenol content of BYVMV infected leaves. Thind *et al.* (1996) recorded a hike in total phenols in the plants infected with yellow mosaic virus as compared to healthy plants due to virus infection. Increased content of both total phenol and *ortho-* dihydroxy phenol (OD-phenol) in *Tomato spotted wilt virus* (TSWV) infected plants was reported by Sutha *et al.* (1997). Higher

content of phenol and flavanol due to *Cotton leaf curl virus* infection was reported in resistant cultivars with respect to susceptible varieties (Kaur *et al.*, 1998). Raghavendra (2002) reported post infection increase in phenols (34.21%) in the plants infected with TLCV. Vasanthy and Shanmugam (2003) observed lowest contents of total phenol and OD-Phenol in healthy sett-propagated cassava as compared to ICMV infected sett-propagated and meristem derived cassava regenerants. Parashar and Lodha (2007) concluded that the resistance in *Foeniculum vulgare* against fungal and viral diseases was attributed to the presence of high amount of phenol. Meena *et al.* (2008) stated that the phenol synthesizing pathways are accelerated after the pathogen attack and it results in the increased levels of phenols. Saveetha *et al.* (2010) while studying the physiological alterations due to viral infection, observed increased phenol contents in *Eleusine coracana* (finger millet) plants infected by *Mottle streak virus*. An increase in phenol content in virus infected plants might be due to increase in HMP shunt pathway which produced intermediaries required for the synthesis of phenolic compounds due to virus infection.

The total soluble protein content was estimated in virus inoculated and healthy ginger plants as per the procedure described by Bradford (1976). The results showed lower protein content in infected plants in comparison with the healthy plants. Also, the healthy plants showed an increasing trend and the infected plants showed a decreasing trend in the total soluble protein content. The present investigation was in accordance with the findings of Johri and Pandhi (1985). They reported that the total protein content in okra declined in diseased tissues due to *Yellow vein mosaic virus* infection. Ahmed *et al.* (1992) studied the effect of yellow vein mosaic disease on the physiology of okra and reported that the total protein and soluble proteins were high in virus – free resistant varieties of okra. Similar finding was given by Thind *et al.* (1996). They reported that *Mungbean yellow mosaic virus* (MYMV) infection on mungbean lowered the protein content in the leaves. Banerjee and Kalloo (1998) recorded high crude protein content in highly TLCV resistant lines of tomato as

compared to susceptible varieties. A reduction in amino acid content of 50 and 77 per cent at 12 and 20 days after infection, respectively was reported in the leaves of *Bean yellow mosaic virus* (BYMV) infected *Phaseolus vulgaris* (Hemida, 2005). Taiwo *et al.* (2007) demonstrated that the protein content was lowered in all the cowpea cultivars and lines as a result of individual as well as mixed infection of *Cowpea aphid borne mosaic virus* (CABMV), *Cowpea mottle virus* (CPMoV) and *Southern bean mosaic virus* (SBMV). Lower protein contents in mesta plants infected with yellow vein mosaic compared to control was reported by Arpita and Subrata (2008). The reason for reduction in total soluble protein in mesta was attributed to yellow vein disease (Chatterjee and Ghosh, 2008). In accordance to this finding, Philip (2010) reported a reduction in total soluble protein content of cassava plants due to *Cassava mosaic virus* (CsMV) infection. All these reports are in conformity with those obtained in the present investigation. The decrease in total soluble protein content of virus infected plants might be due to the exploitation of protein synthesis machinery of the host by the virus. The viruses lack their own protein synthesizing mechanisms and they do depend on their hosts for the same. Hence this might have resulted in the decline in protein content of the host plants, with respect to that of non infected plants.

Activity of defense related enzymes such as Peroxidase (PO), Polyphenol oxidase (PPO) and Phenylalanine ammonialyase (PAL) were also analyzed at different stages of infection. Defense related enzymes were reported to act as an important factor in the induction of resistance (Dasgupta, 1988).

Peroxidase activity was more in infected plants than that of healthy plants at all stages of infection. The PO activity was observed to be increasing except at 120 DAI. PO activity was at the peak on 90 DAI in infected plants (54.50 min^{-1} g^{-1}). Similarly PPO activity was also found higher in infected plants than in healthy plants. PPO activity was at the peak on 90 DAI in infected plants (13.43 min^{-1} g^{-1}). The activity was in an increasing trend up to 90 DAI and then declined to 3.07 min^{-1} g^{-1} at 120 DAI. PAL, another important enzyme was more active in infected plants except

at 30DAI, where the healthy as well as infected plants expressed PAL activities which are not significantly different (95.83 $\mu g\ g^{-1}$ and 90.56 $\mu g\ g^{-1}$ respectively).

Khatri and Chenulu (1970) observed significantly higher PO activity in virus infected cowpea. Similarly, Ramiah *et al.* (1973) reported that peroxidase (PO) activity increased in the leaves of BYVMV infected plants. Batra and Kuhn (1975) found two to three times increased polyphenol oxidase and peroxidase activity in primary leaves of hypersensitive soybean plants infected with *Cowpea chlorotic mottle virus*. Wagih and Coutts (1982) reported that *Tobacco necrosis virus* infected cowpea and cucumber showed increase in peroxidase and polyphenol oxidase activity. Sharma *et al.* (1984) showed the effect of viral and fungal infections in musk melon and reported an increasing trend of enzyme activity when compared to healthy control. Sindhu (2001) studied on the changes of defense related enzymes such as PO, PPO and PAL and stated that there was significant increase in activities of these enzymes in inoculated cowpea plants. Clarke *et al.* (2002) and Karthikeyan *et al.* (2007) also observed significant increase in PO activity in *Phaseolus vulgaris* and *Vigna mungo* after *White clover mosaic virus* (WClMV) inoculation and *Urdbean leaf crinkle virus* (ULCV) inoculation, respectively. Veena (2007) observed an increase in the three defence related enzymes (PO, PPO and PAL) in AVP treated cowpea plants challenged with BlCMV compared to that of the healthy and virus inoculated cowpea plants. Meena *et al.* (2008) observed high PPO activity in geminivirus infected chilly leaf as compared to healthy. Tomato plants exhibited more PO activity in the presence of TLCV and the activity of the enzyme was found higher in mature than in juvenile leaves (Dieng *et al.*, 2011). Krishnapriya (2015) reported a higher peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) activity for BlCMV inoculated cowpea plants compared to healthy plants. Increased peroxidase activity was associated with resistance reaction which could be due to increased phenol concentration, where phenols were co-factor of peroxidase and hence influenced resistance in the host. The enhanced polyphenol oxidase activity might result in the augmented rate of oxidation of phenolics substance that participates in

the defence reaction of host. PAL is the enzyme which catalyzes conversion of L-Phenylalanine to *trans-* cinnamic acid. This is the first and promising step of phenyl propanoid pathway in plants. Phenyl propanoid pathway results in formation of phenols, lignin, isoflavonoids etc. which are having major roles in inducing systemic resistance in plants.

Viral infection leads to the appearance of specific soluble proteins. The protein profile analysis of young leaves of the healthy and infected cassava plants using SDS- PAGE showed the presence of an additional protein band in infected samples and the molecular weight of this additional band was estimated as 20 kDa using protein markers. Molecular weight of the extra band revealed the presence of an induced protein in infected samples, which are assumed to be induced in the plant due to the virus infection. Nambisan (1996) observed that these new proteins are similar to some low molecular weight proteins produced in response to stress.

Isozyme analysis of defense related enzyme PO using native PAGE produced an isoform with relative mobility (R_m) value of 0.812 in infected sample. Rathi *et al.*, (1986) reported that the resistance against sterility mosaic virus in pigeon pea cultivars was attributed to specific isoperoxidase and proteins. Arpita and Subrata (2008) reported isozyme analysis of peroxidase by native PAGE, which showed alteration in the activities of different enzymes due to infection by yellow vein mosaic. Meena *et al.* (2008) conducted the electrophoretic studies in chilly infected with geminivirus and revealed the presence of three iso peroxidase bands in diseased leaves, whereas two in healthy leaves. The existence of isozymes is the signal of fine tuning of metabolism to meet the particular needs of the tissue. The present findings are in accordance with the previous reports on the isozyme studies.

5.4. Immunological diagnosis

DAC- ELISA using the polyclonal antibodies specific to *Banana bract mosaic virus* (BBrMV), *Cucumber mosaic virus* (CMV), *Sugarcane bacilliform virus* (SCBV), *Southern bean mosaic virus* (SBMV) and *Tobacco mosaic virus* (TMV) was carried out to detect the presence of the above viruses. TAS- ELISA was carried out

for detecting the presence of begomoviruses using the polyclonal antibodies available against begomoviruses, *African cassava mosaic virus* (ACMV) and *Sri Lankan cassava mosaic virus* (SLCMV). From the ELISA reactions the antibodies against BBrMV, CMV and ACMV reacted positively. These were again confirmed using Dot immunobinding assay (DIBA), in which BBrMV gave positive result. ACMV antibody did not respond to DIBA reaction. From the literature available, So (1980) stated that a virus isolate from ginger plants showing mosaic symptoms gave positive serological reaction with antiserum against *Cucumber mosaic virus* (CMV). Thomas (1986) suggested that the newly observed *Ginger chlorotic fleck virus* (GCFV) reflected its affinities to the sobemovirus group. Hence the antibody against SBMV was tested in DAC- ELISA. Many properties of the members of sobemovirus group are reported to be serologically unrelated by Seghal (1981). Conforming to the findings of Seghal, Thomas could not confirm the positive serological reaction with the antisera against several sobemoviruses. Similarly, the present study also could not get a positive reaction of SBMV in ELISA. Thomas (1986) also tested the reaction of purified GCFV with the antisera against many plant viruses including CMV, *Sowbane mosaic virus*, *Tobacco necrosis virus*, *Tobacco mottle virus* and SBMV, in which he reported negative reaction with all of the antisera.

A virus isolate from ginger in China was identified serologically positive against *Tobacco mosaic virus* (TMV) by Fan *et al.* (1999). In the present study also, it was serologically negative. Zhang *et al.* (2016) carried out DAS- ELISA in flowering ginger (*Alpinia purpurata*) using the antiserum of *Banana bract mosaic virus* (BBrMV). In the assay they reported positive reaction and detected BBrMV in *A. purpurata*. This finding is in agreement with the present results.

5.5. Molecular diagnosis and sequencing

Molecular diagnosis using Polymerse chain reaction (PCR) was performed for the detection of begomoviruses, where RT- PCR was performed for the detection of BBrMV genomes. A pair of degenerate primers for begomovirus genus and a pair for Potyviruses were used for the detection of the respective viruses. Primer pair specific

for coat protein gene was used for the detection of BBrMV and BBrMV isolate from flowering ginger. An amplicon of size 550 bp was obtained in begomovirus degenerate primer (AV494- F and AC1048- R). None of the other primers gave any positive reaction in the PCR.

Siljo *et al.* (2012) identified that the chlorotic streak- infected cardamom plants tested negative in RT- PCR for *Cardamom mosaic virus* (CdMV). They performed RT- PCR again using the primers targeted to conserved regions of *Potyvirus*. It gave approximately 700 bp amplicon, upon sequencing and BLAST analysis showed *Banana bract mosaic virus* (BBrMV) as the most related virus. Cloning and sequencing of the coat protein gene of the virus isolates from different geographical regions was carried out using primers specific for BBrMV, which gave positive results. Zhang *et al.* (2016) achieved deep sequencing of BBrMV from flowering ginger (*Alpinia purpurata*) and developed an immunocapture reverse transcription loop- mediated isothermal amplification (IC-RT-LAMP) assay. Based on these studies BBrMV primers were used for the study and it gave negative result. The present study could amplify only in PCR with degenerate primers for begomoviruses.

The PCR products were sequenced at AgriGenome Labs private limited, Smart City, Kochi. The sequence was analyzed using BLAST software and 17 superior sequences were selected for cluster dendrogram construction. Dendrogram revealed that the following were the nearest related sequences:

1) *Tomato leaf curl Bangalore virus* from India coat protein (AV1) gene, partial cds; replication associated protein (AC3) and transactivator protein (AC2) genes, complete cds; and replicase (AC1) gene, partial cds

2) *Tomato leaf curl Bangalore virus* from India coat protein (AV1) gene, partial cds; replication associated protein (AC3) and transactivator protein (AC2) genes, complete cds; and replicase (AC1) gene, partial cds

These sequences were observed to have 71% and 73% of sequence similarity with the virus under present investigation.

Since the sequence similarities are very low in per cent, and the multiple positive results expressed in ELISA, the virus under investigation can be subjected to further future studies and the possibilities for naming the virus as a new viral species is also not so far.

5.6. Management of the disease

The experiment was conducted in completely randomized design (CRD) with 13 treatments and three replications. Karthika was the variety used for the study. Vulnerability index (V.I.) was calculated after application of treatments, as described by Bos (1982). Three sets of sprays were given at fortnightly interval. V.I.. before first, second and third sprays and two weeks after third spray were calculated. The V.I. values calculated before first spray were ranging from 35.66 to 48.67, which were not significantly different. After the first spray, V.I. values were calculated just before the second spray. It was observed that the least V.I. was estimated from the plants treated with Perfekt at 1.0 ml L^{-1}(V.I.. 37.09) and 10% leaf extract of *Mirabilis jalapa* (V.I.. 37.03). These treatments were found statistically on par.

The V.I. values estimated before third spray revealed that Perfekt at 0.5 ml L^{-1} (V.I.. 37.90), Perfekt at 1.0 ml L^{-1}(V.I.. 37.07) and 10% *M. jalapa* (V.I.. 37.10) were superior than the other treatments. After two weeks of the third spray V.I. values were calculated. Among the values, the least values were 30.66 and 31.42, from which the treatments with Perfekt at 0.5 ml L^{-1} and 1.0 ml L^{-1} were applied respectively. The second best treatment was observed to be 10% leaf extract of *M. jalapa* with a V.I. value of 38.15.

Similar results were obtained by Verma and Kumar (1980). They reported that the leaf extracts of *Datura* spp., *Azadirachta indica* and *Mirabilis jalapa* contained antiviral proteins which repressed infection by CMV, TMV and yellow vein mosaic virus in black gram. Habuka *et al.* (1991) found that antiviral principles isolated from roots of *Mirabilis jalapa* inhibited mechanical transmission of plant viruses. Vivanco *et al.* (1999) evaluated extracts of *Mirabilis jalapa* containing a ribosome inactivating protein (RIP) called mirabilis antiviral protein (MAP), against infection by *Potato*

virus X, Potato virus Y, Potato leaf roll virus and *Potato spindle tuber viroid*. They found there were remarkable reductions in viral incidence. Renukadevi *et al*. (2004) reported that the AVP from *M. jalapa* (MAP) and *Herpula cupanioides* (HAP) were highly effective in inhibiting TSWV. Pre-application of MAP and HAP induced the activity of phenols, peroxidise (PO), polyphenol oxidase (PPO) and phenylalanine ammonialyase (PAL) leading to the suppression of TSWV on local lesion and systemic host.

Similarly, the leaf extract of *Bougainvillea spectabilis* was effective in inhibiting infection of the virus to a significant level of 54.72% inhibition. Sreelekha (1987) revealed that cowpea mosaic infection could be effectively controlled by pre inoculation spraying of *Bougainvillea* spp., which is in agreement with the present results. Similar results were reported by Balasaraswathy *et al*. (1998). They suggested that foliar spray of leaf extract of *Bougainvillea spectabilis* was effective in inhibiting infection of TSWV, TMV, CMV and CaMV.

The application of chemicals namely Aspirin, salicylic acid and barium chloride could inhibit the expression of symptoms and the final V.I. values expressed by the treated plants ranged from 46.78 to 52.28, whereas the untreated control plants showed a V.I. of 100. White (1979) reported that pathogenesis related (PR) protein accumulation and resistance to Tobacco mosaic virus (TMV) could be induced by treatment of tobacco with salicylic acid (SA), aspirin (acetyl SA or ASA) or benzoic acid (BA). Singh *et al*. (2004) reported multiple antiviral defence mechanism of SA. They observed that SA triggers resistance to viral infection process such as replication, cell to cell movement and long distance movement.

White *et al*. (1986) reported that the chemicals manganese chloride and barium chloride induced resistance in Xanthi-nc tobacco leaves to TMV infection. Bioassy of chemicals such as SA, manganese chloride and barium chloride to evaluate their efficiency in reducing the symptoms caused by *Cowpea aphid borne mosaic virus* (CABMV) in *Chenopodium amaranticolor* and cowpea revealed that the post-inoculation treatment of manganese chloride gave maximum inhibition of

symptoms (Radhika, 1999). Pun *et al*. (2000) reported that barium chloride was most effective in reducing the symptoms of *Bhindi yellow vein mosaic virus* (BYVMV) when sprayed exogenously on okra plants.

The present study on the mangement of the disease found that botanical extracts, namely Perfekt and 10% leaf extract of *Mirabilis jalapa* were most effective. The antiviral chemicals gave almost 50 per cent reduction in V.I. as compared to the untreated control plants, which are in conformity with the previous studies.

Summary

6. SUMMARY

Economically significant and rhizome transmitted virus of ginger causing mosaic disease in ginger was selected for the study owing to their widespread occurrence in ginger growing tracts of Kerala. The present study entitled "Etiology and management of mosaic disease in ginger (*Zingiber officinale* Rosc.) was carried out at the department of Plant Pathology, College of Agriculture, Vellayani during 2015-2018, with the objectives to identify, characterize, clone and sequence the genes of *Ginger mosaic virus* infecting ginger along with the management of the disease using antiviral principles of plant, chemical and microbial origin.

As part of the study, infected rhizomes were collected and grown in pots. The plants which emerged from those rhizomes were observed for the appearance of symptoms. The symptoms were observed as small light green flecks on newly emerged leaves. These flecks gradually increased in size and eventually formed streaks. The streaks were arranged parallel to the veins. The appearance of too many streaks on the leaves led to severe chlorosis and the leaves showed necrotic symptoms in advanced stage.

The seed material (rhizomes) was collected from infected plants in the campus. Transmission of the virus was tested in rhizomes as well as in mechanical inoculation to healthy plants. Rhizome transmission was tested by planting the infected rhizomes. Mechanical inoculation was performed using infected leaf sap. The sap was inoculated to healthy leaves, after dusting with carborundum powder. The infected rhizomes resulted in 100% transmission and mechanical transmission failed to transmit the virus.

The physiological changes in ginger due to viral infection were studied at 30, 60, 90 and 120 days after infection. It comprised of estimating changes in total carbohydrates, chlorophyll, phenol, protein and analysis of defense related enzymes such as peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonialyase (PAL). The study revealed an increase in the contents of phenols and defense related enzymes in infected plants than that of healthy plants. Total

carbohydrates, chlorophyll and protein contents were observed more in healthy plants as compared to that of infected plants.

Protein profile and isozyme analysis in healthy and diseased plants were carried out using Sodium dodecyl sulphate (SDS) and native Polyacrylamide gel electrophoresis (PAGE), respectively. SDS- PAGE indicated the presence of a novel protein with molecular weight of 20 kDa in the infected sample. Isozyme analysis revealed that there was an extra activity of peroxidase in infected sample. The peroxidase activity in the virus infected samples showed the presence of definite single band with Rm value of 0.812.

The immunological detection techniques direct antigen coating- enzyme linked immunosorbant assay (DAC-ELISA) and dot immunobinding assay (DIBA) were carried out. Since the etiology of the virus was unknown, seven suspected viruses namely *Banana bract mosaic virus* (BBrMV), *Cucumber mosaic virus* (CMV), *Tobacco mosaic virus* (TMV), *Sugarcane bacilliform virus* (SCBV), *Southern bean mosaic virus* (SBMV), *African cassava mosaic virus* (ACMV) and *Sri Lankan cassava mosaic virus* (SLCMV) were tested. The tests using DAC-ELISA were positive to antibodies specific to two viruses namely *Banana bract mosaic virus* (BBrMV), *Cucumber mosaic virus* (CMV). These antibodies gave an absorbance value of 0.15 and 0.19 respectively which was three times more than the absorbance shown by the healthy sample (0.05 and 0.07). The infected leaf tested for the presence of *African cassava mosaic virus* (ACMV) by triple antibody sandwich ELISA (TAS- ELISA) gave an absorbance of 0.0425 while the healthy gave an absorbance of 0.017. DIBA analysis gave positive reaction to BBrMV.

Polymerase chain reaction (PCR) was carried out with both total DNA and RNA isolated from infected ginger leaf sample. The PCR experiment was positive to Begomoviruses and negative to potyviruses, BBrMV and CMV isolates. An amplicon of size 550 bp was obtained for the sample DNA using Begomo degenerate primer. The sequence was subjected to BLAST analysis which indicated 74% similarity to *Tomato leaf curl virus Bangalore isolate*. The sequence data was analyzed, compared and alignments were made using

CLUSTAL-W software. Sequence phylogram was constructed using PHYLIP package and unrooted trees were generated using TREEVIEW software.

The management studies were carried out with antiviral principles like botanicals, chemicals and that of microbial origin against the virus. The experiment was conducted in completely randomized design (CRD) with 13 treatments in three replications. Karthika was the variety used for the study. Perfekt (a botanical extract-76%) at 0.5 ml L^{-1} and 1 ml L^{-1}, chemicals namely aspirin, salicylic acid, barium chloride at 100 and 150 ppm concentrations, botanicals namely 10% leaf extracts of *Mirabilis jalapa* and *Bougainvillea spectabilis,* 2% Neem oil- garlic emulsion and 2% PGPR mix II were used in the experiment. The treatments were given at fortnightly intervals. Before each spray the efficacy of treatments were evaluated using Vulnerability Index (V.I.) developed by Bos (1982). The treatments with Perfekt at 0.5 ml L^{-1} and 1 ml L^{-1} and 10% leaf extract of *Mirabilis jalapa* were found effective for the management of the disease.

References

7. REFERENCES

Ahmed, N., Thakur, M. R. and Bajaj, K. L. 1986. Nature of resistance and effect of *Yellow vein mosaic virus* on moisture, chlorophyll, chlorophyllase and carbohydrate contents of okra. *Veg. Sci.* 13: 339-353.

Ahmed, N., Thakur, M. R., Bajaj, K. L. and Cheema, S. S. 1992. Biochemical basis of resistance to *Yellow vein mosaic virus* in okra. *Plant Dis. Res.* 9: 20-25.

Aiyanathan, E.A.K and Narayanaswamy, P. 1988. Effects of neem oil on RTV infection in rice varieties with different levels of resistance. Proc. Natl. Sem. Management of Crop Diseases with Plant Products/ Biological agents., Tamil Nadu Agricultural University, Madurai, p.40.

Alagianagalingam, M. N. and Ramakrishnan, K. 1979. Effect of cassava mosaic on photosynthetic pigments and photosynthesis. *Auara.* 7: 120-123.

Alex, T. 2017. Exploration of natural products from botanicals and root endophytes for the management of *Cowpea mosaic virus*. M. Sc. (Ag.) Thesis, Kerala Agricultural University, Thrissur. 147 p.

[Anonymous]. 2016. *National Horticulture Board* [online]. Available: http://www.nhb.gov.in

Arpita, C. and Subrata, K. G. 2008. Alterations in biochemical components in mesta plants infected with yellow vein mosaic disease. *Braz. J. Pl. Physiol.* 20: 267-275.

Ashfaq, M., Khan, A. M., Javed, N., Mughal, S. M, Shahid, M. and Sahi, S. T. 2010. Effect of Urd bean crinkle virus infection on total soluble protein and anti oxidant enzymes in black gram plants. *Pak. J. Bot.* 42: 447-454.

Ayanru, D. K. G. and Sharma, V. C. 1982. Effects of cassava mosaic disease on certain leaf parameters of field grown cassava and clones. *Phytopath.* 72: 1057-1059.

Balasaraswathy, R., Sadasivam, S., Ward, M. and Walker, J. M. 1998. Antiviral protein from *Bougainvillea sprctabilis* roots; purification and characterization. *Phytochem.* 47: 1561-1565.

Banerjee, M. K. and Kalloo, G. 1998. Sources and inheritance of resistance to leaf curl in *Lycopersicon*. *Theory Appl. Genet.* 73: 707-710.

Batra, G. K. and Kuhn, C. W. 1975. Polyphenol oxidase and peroxidase activities associated with acquired resistance and its inhibition by 2-thiouracil in virus infected soybean. *Physiol. Pl. Path.*, 5: 239-248.

Bensal, R. D., Sandhu, P. S., Tarsemlal and Sandhu, K. S. 1990. Effect of antiviral substances on some biochemical constituents in response to cucumber mosaic infection in summer squash. *Ann. Pl. Protec.* 7: 163-165.

Bhagat, A. P. and Yadav, B. P. 1997. Biochemical changes in BYVMV infected leaves of bhendi. *J. Mycol. Pl. Pathol.* 27: 94-95.

Bhatia, S., Kapoor, H. C. and Lodha, M. L. 2004. Modification of Antioxidant Status of Host Cell in Response to *Bougainvillea* Antiviral Proteins. *J. Pl. Biochem. Biotech.*, 13: 113-118.

Bos, L. 1982. Crop losses caused by viruses. *Adv. Virus Research.*, 2: 31-57.

Bradford, M. M. 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principles of protein dye- binding. *Ann. Biochem.* 72: 248.

Bray, G. G. and Thorpe, W. V. 1954. Analysis of phenolic compounds of interest in metabolism. *Methods Biochem. Anal.* 1: 27-52.

Carl, M. N., Chung, L. K., Catherine, A. M, Wong, S. K. and Carr, J. P. 2005. Salicylic acid-introduced resistance to *Cucumber mosaic virus* in squash and *Arabidopsis thaliana*: Contrasting mechanisms of introduction and antiviral action. *Mol. Pl. Microb. Interact.*, 18: 428-434.

Chatterjee, A. and Ghosh, S. K. 2008. Alterations in biochemical components in Mesta plants infected with yellow vein mosaic disease. *Brazilian J. Plant Physiol.* 20(4): 267-275.

Chen, C., Belanger, R. R., Benhamou, N. and Paultiz, T. C. 2000. Defence enzymes induced in cucumber roots by treatment with plant growth promoting rhizobacteria (PGPR). *Physiol. Mol. Pl. Path.*, 56: 13-23.

Chivasa, S., Murphy, A. M., Naylor, M. and Carr, J. P. 1997. Salicylic acid interferes with *Tobacco mosaic virus* replication via a novel salicylhydroxamic acid-sensitive mechanism. *Pl. Cell.*, 9: 547-557.

Choudhuri, A. K. and Saha, N. K. 1985. Inhibition of *Urdbean leaf crinkle virus* by different plant extracts. *Indian Phytopath.*, 38: 566-568.

Clarke, S. F., Guy, P. L., Burrit, D. J. and Jaseson, P. E. 2002. Changes in the activities of anti oxidant enzymes in response to virus infection and hormone treatment. *Physiologia Plant.* 114: 157-164.

Conti, G. G., Bassi, M., Dolce, L. and Barbieri, N. 1988. Morphological alterations induced by acetylsalicylic acid in *Datura* plants. *Cytobios.*, 54: 7-16.

Dasgupta, M. K. 1988. *Principles of Plant Pathology.* Allied Publishers Private Limited, New Delhi, 1040 p.

Dempsey, D., Shah, J. and Klessig, D. F. 1999. Salicylic acid and disease resistance in plants. *Crit. Rev. Pl. Sci.,* 18: 547-575.

Dickerson, D. P., Pascholati, S. F., Hagerman, A. K., Butler, L. G. and Nicholson, R. L. 1984. Phenylalanine ammonia lyase hydroxyl cinnamate: COA maydis or Ligase in maize mesocotyls inoculated with *Helminthosporium maydis* or *Helminthosporium carbonum*. *Physiol. Pl. Pathol.* 25: 111-123.

Dieng, H., Satho, T., Hassan, A. A., Aziz, A. T., Morales, R. E., Ab Hamid, S., Miake, F. and Abubakar, S. 2011. Peroxidase activity after viral infection and whitefly infestation in juvenile and mature leaves of *Solanum lycopersicanum*. *J. Phytopath.* 159: 707-712.

Durrant, W. E. and Dong, X. 2004. Systemic acquired resistance. *Ann. Rev. Phytopath.*, 42: 185-209.

Estellita, S., Suma, A., Luckins, C. B. and Mallika, V. K. 1993. Flower bud anatomy of kokkan affected banana. *J. Tropic Agric.*31: 277-278.

Fan, G. Q., Wang, J. Y., Guo, X. Q. and Zhu, H. C. 1999. Identification of *Tobacco mosaic virus* infecting ginger. *J. Shandong Agric. Univ.* 30 (3): 249-255.

Gruner, R., Strompen, G., Pfitzner, A.J.P. and Pfitzner, U.M. 2003. Salicylic acid and the hypersensitive response initiate distinct signal transducrtion pathways in tobacco that converge on the as-1-like element of the PR-1a promoter. *Eur. J. Biochem.,* 270: 4876-4886.

Habuka, N., Miyano, M., Kataoka, J. and Noma, M. 1991. *Escherichia coli* ribosome is inactivated by *Mirabilis* antiviral protein, which cleaves the N-glycosidic bond at A 2660 of 23S Ribosomal RNA. *J. Mol. Biol.,* 291: 127-131.

Hedge, J. E. and Hoftreiter, B. T. 1962. Methods. In: Whistler, R. L. and Be Miller, J. N. (eds.), *Carbohydrate Chemistry.* Academic Press, New York, p. 420.

Hemida, S. K. 2005. Effect of *Bean yellow mosaic virus* on physiological parameters of *Vicia faba* and *Phaseolus vulgaris*. *Int. J. Agr. Biol.* 7(2): 154-157.

Idris, A. M., Hiebert, E., Bird, J. and Brown, J. K. 2003. Two newly described Begomoviruses of *Macroptilium lathyroides* and Common bean. *Phytopathol.* 93: 774-783.

Jacob, T. and Usha, R. 2001. 3′-Terminal Sequence Analysis of the RNA Genome of the Indian Isolate of Cardamom Mosaic Virus: A New Member of Genus *Macluravirus* of *Potyviridae*. *Virus Genes.* 23: 81-88.

Johri, J. K. and Pandhi, B. 1985. Effect of yellow vein mosaic on physiology of okra. *Indian J. Virol.* 1: 61-68.

Kanchalee, J., William, F. D. and Kloepper, J. W. 2003. Broad spectrum protection against several pathogens by PGPR mixtures under field conditions in *Thailand. Pl. Dis.* 87: 1390-1394.

Karthikeyan, G., Doraisamy, S. and Rabindran, R. 2007. Induction of systemic resistance in black gram against *Urd bean leaf crinkle virus* by chemicals. *Arch. Phytopath. Pl. Protec.* 40: 1-15.

Kaur, G., Sohal, B. S., Singh, J. and Bajaj, K. L. 1998. Influence of *Cotton leaf curl virus* on the polyphenol metabolism of resistant and susceptible cotton leaves. *Pl. Dis. Res.*, 13: 23-27.

Khan, S., Jan, A. T., Mandal, B. and Haq, Q. R. 2011. Immunodiagnosis of *Cucumber mosaic virus* using antisera developed against recombinant coat protein. *Arch. Phytopathol. and Pl. Protec.* 45(5): 561-569.

Khatri, H. L. and Chenulu, V. V. 1970. Metabolism of resistant and susceptible cowpea varieties infected with cowpea mosaic virus. II changes in peroxidase and catalase enzyme activity. *Indian Phytopath.* , 23: 553-557.

Krishnapriya, P. J. 2015. Immunomolecular detection and characterization of potyviruses infecting cowpea (*Vigna unguiculata* (L.) Walp.) and papaya (*Carica papaya* L.). M. Sc (Ag). Thesis, Kerala Agricultural University, Thrissur., 198 p.

Lamelli, O. K. 1970. Cleavage of structural proteins during the assembly of head of bacteriophage T4. *Nat.* 227: 680-685.

Liou, R. F., Yan, H. Z. and Hong, J. L. 2003. Molecular evidence that aphid-transmitted *Alpinia mosaic virus* is a tentative member of the genus *Macluravirus*. *Arch. Virol.*, 148: 1211-1218.

Lodhi, A. M., Guang-Ning, Y., Weeden, N. F. and Reisch, B. I. 1994. A simple and efficient method for DNA extraction from grapevine cultivars and *Vitis* species. *Pl. Mol. Biol. Reporter.* 12: 6-13.

Lopez, C., Ferriol, M. and Pico, M. B. 2015. Mechanical transmission of *Tomata leaf curl New Delhi virus* to cucurbit germplasm: selection of tolerance sources in *Cucumis melo*. *Euphytica.* 10: 107-111.

Magnaye, L. V. and Epsino, R. R. C. 1990. Banana bract mosaic- a new disease of banana. *Philippine Agriculturist.* 73: 55-59.

Mali, P. C., Burman, U. and Lodha, S. 2000. Effect of planting dates and development of yellow mosaic virus on biochemical constituents of moth bean genotypes. *Indian Phytopath.* 53: 377-383.

Mariappan, V., Jayaraj, S. and Saxena, R. C. 1988. Effects of non-edible seed oils on survival of *Nephotettix virescens* (Homoptera: Cicadellidae) and on transmission of rice tungro virus. *J. Econ. Entomol.,* 81: 1369-1372.

Maurhofer, M., Hase, C., Meuwly, P., Metraux, J. P. and Defago, G. 1994. Induction of systemic resistance of tobacco to *Tobacco mosaic virus* by the root-

colonizing *Pseudomonas fluorescens* strain CHAO: Influence of the gacA and of pyoverdine production. *Phytopath.*, 84: 139-146.

Mayer, A. M., Harel, E. and Shaul, R. B. 1965. Assay of catechol oxidase, a critical comparison of methods. *Phytochem.* 5: 783-789.

Meena, R. K., Vidya, P. and Arora, D. K. 2008. Study on phenolics and their oxidative enzymes in *Capsicum annum* L. infected with geminivirus. *Asian J. Exp. Sci.*, 22: 307-310.

Momol, T. M. and Pernezny, K. L. 2006. Specific common diseases. Florida Plant Disease Management Guide Everglades Research and Education Centre. University of Florida. Florida Cooperative Extension Service, IFAS. *PDMG.* 3: 53.

Mosch, J., Mende, A., Zeller, W., Rieck, M. and Ullrich, W. 1993. Plant extracts with a resistance induction effect against fire blight (*Erwinia amylovora*). *Acta Hort.*, 338-395.

Murphy, A. M,, Gilliland, A., Wong, C. E., West, J., Singh, D. P. and Carr, J. P. 2001. Signal transduction in resistance to plant viruses. *Eur. J. Pl. Path.*, 107: 121-128.

Murphy, A. M. and Carr, J. P. 2002. Salicylic acid has cell-specific effects on *Tobacco mosaic virus* replication and cell-to cell movement. *Pl. Physiol.*, 128: 552-563.

Murphy, J. F., Reddy, M. S., Ryu, C. M., Kloepper, J. W. and Li, R. 2003. Rhizobacteria-mediated growth promotion of tomato leads to protection against *Cucumber mosaic virus*. *Phytopath.*, 93: 1301-1307.

Murphy, J. F., Zehnder, G. W., Schuster, D. J., Sikora, E. J., Polston, J. E. and Kloepper, J. W. 2000. Plant growth-promoting rhizobacterial mediated protection in tomato against *Tomato mottle virus*. *Pl. Dis.*, 84: 779-784.

Nambiar, K. K. and Sarma, Y. R. 1974. Mosaic disease in ginger. *Arecanut and Spices Bull.*, 6: 3-4.

Nambisan, B. 1996. Pathogenesis- related proteins in cassava mosaic disease. In: Kurup, G. T., Palaniswamy, M. S., Potty, V. P., Padmaja, G., Kabeerathumma, S. and Pillai, S. V. (eds.), *Tropical tuber crops: problems, prospects and future strategies.* Oxford & IBH Publishing Co., New Delhi., pp. 375-379.

Nandakumar, R., Babu, S., viswanathan, R., raguchander, T. and Samiyappan, R. 2001. Induction of systemic resistance in rice against sheath blight disease by *Pseudomonas fluorescens*. *Soil Biol. Biochem.*, 33: 603-612.

Naylor, M., Murphy, A. M,, Berry, J. O. and Carr, J. P. 1998. Salicylic acid can induce resistance to plant virus movement. *Mol. Pl. Microb. Interact.*, 11: 860-868.

Parashar, A. and Lodha, P. 2007. Phenolics estimation in *Foeniculum vulgare* infected with Ramularia blight. *Ann. Pl. Protec. Sci.* 15: 396-398.

Philip, T. 2010. *Cassava mosaic virus* induced physio- biochemical changes in the leaves of tapioca (*Manihot utilisima* Pohl.). *Int. J. Plant Protec.*, 3: 31-33.

Prasad, H. P., Shankar, U. A. C., Kumar, B. H., Shetty, S. H. and Prakash, H. S. 2007. Management of *Bean common mosaic virus* strain *Blackeye cowpea mosaic* (BCMV-BlCM) in cowpea using plant extracts. *Arch. Phytopath. Pl. Protec.*, 40: 139-147.

Pun, K. B., Sabitha, D. and Jeyarajan, R. 2000. Screening of virus inhibiting chemicals and neem products against *Okra yellow vein mosaic virus*. *Indian Phytopath.*, 53: 95-96.

Radhika, N. S. 1999. Disease resistance in the management of *Cowpea aphid-borne mosaic virus*. M. Sc. Thesis, Kerala Agricultural University, Thrissur., 106p.

Raghavendra, A. S. 2002. Histology, Biochemical studies and management of tomato leaf curl. M. Sc (Ag) thesis, University of Agricultural Sciences, Dharwad, 63 p.

Ramakrishnan, K., Nambiar, K. K. N. and Alagianagalingam, M. N. 1968. Physiology of virus infected plants. *Biomedical Life Sci.* 69: 104-114.

Ramiah, M. 1978. Studies on mosaic disease of cowpea in relation to disease resistance. Ph. D. thesis, Tamil Nadu Agricultural University, Coimbatore, 105p.

Rathi, Y. P. S., Bhatt, A. and Sing, U. S. 1986. Biochemical changes in pigeon pea (*Cajanus cajan*(L) Mill sp.) leaves in relation to resistance against sterility mosaic disease. *J. Biosci.*, 10: 467-474.

Raupach, G. S., Liu, L., Murphy, J. F., Tuzun, S. and Kloepper, J. W. 1996. Induced systemic resistance in cucumber and tomato against cucumber mosaic cucumovirus using plant growth-promoting rhizobacteria (PGPR). *Pl. Dis.*, 80: 891-894.

Rayachaudhuri, S. P. and Ganguly, B. 1965. Further studies on chirke disease of Large Cardamom (*Amomum subulatum* Roxb.). *Indian Phytopathol.*, 18: 373-377.

Renukadevi, P., Doraiswamy, S., Nakkeeran, S., Rabindran, R., Ganapathy, T., Ramiah, M. and Mathiyazhagan, S. 2004. Antiviral action of *Herpulia*

cupanioides and *Mirabilis jalapa* against *Tomato spotted wilt virus* (TSWV) infecting tomato. *Arch. Phytopath. Pl. Protec.*, 37: 245-259.

Rodoni, B. C., Dale, J. L. and Harding, R. M. 1999. Characterization and expression of the coat protein-coding region of banana bract mosaic potyvirus, development of diagnostic assays and detection of the virus in banana plants. *Arch. Virol.* 144(9): 1725-1737.

Roychoudhuri, R. and Jain, R. K. 1993. Neem for the control of aphid and whitefly vectors and virus diseases of plants. In: *Neem and Environment*. Singh, R. P., Chari, M. S., Raheja, A. K. and Kraus, W (eds). Oxford and IBH Publishing Co. pp: 763-775.

Ryu, C. M., Murphy, J. F., Kirankumar, S. M. and Kloepper, J. W. 2004. Plant growth-promoting rhizobacteria systemically protect *Arabidopsis thaliana* against *Cucumber mosaic virus* by a salicylic acid and NPRI-independent and jasmonic acid-dependent signalling pathway. *Pl. J.*, 39: 381-392.

Sarma, U. C., Bhagabati, K. N. and Sarkar, C. R. 1995. Effect of yellow vein mosaic virus infection on some chemical constituents of bhendi (*Abelmoschus esculentus* (L.) Moench). *Indian J. Virol.* 11: 81-83.

Sastry, K. S. and Nayudu, M. V. 1988. Studies on biochemical changes in cowpea (Vigna unguiculata (L.) Walp.) infected with *Tobacco ringspot virus*. *Indian J. Virol.* 4: 138-139.

Saveetha, K., Sankaralingam, A. and Muthulakshmi, P. 2010. Changes in physiology and biochemistry of mottle streak virus infected finger millet plants. *Arch. Phytopath. Pl. Protec.* 43: 1273-1285.

Schneider, S. and Ullrich, W. R. 1994. Differential induction of resistance and enhanced enzyme activities in cucumber and tobacco caused by treatment with various abiotic and biotic inducers. *Physiol. Mol. Pl. Path.*, 45: 291-304.

Seghal, O.P. 1981. Southern bean mosaic virus group. In: *Handbook of Plant Virus Infections: Comparative Diagnosis*., Ed. E. Kurstak, Amsterdam., Elsevier/ North-Holland Biomedical Press. pp: 91-121.

Sharma, O. P., Khatri, H. L. and Bansal, R. D. 1984. Effect of *Cucumber mosaic virus* and/ or *Sphaerotheca fulginea* on phenolics, peroxidase and polyphenol oxidase content in musk melon. *Indian J. Mycol. Pl. Path.* 14: 107-111.

Siljo, A., Bhat, A. I., Biju, C. N. and Venugopal, M. N. 2012. Occurrence of *Banana bract mosaic virus* on cardamom. *Phytoparasitica.*, 40: 77-85.

Sindhu, A. R. 2001. Biochemical basis of resistance against *Blackeye cowpea mosaic virus* in cowpea (Vigna unguiculata (L.) Walp.). M. Sc (Ag) thesis, Kerala Agricultural University, Thrissur., 65 p.

Singh, D. P., Catherine, A. M., Gilliland, A. and Carr, J. P. 2004. Activation of multiple antiviral defence mechanisms by salicylic acid. *Mol. Pl. Path.*, 5: 57-63.

Singh, R. and Singh, A. K. 1980. Metabolic changes in leaf tissue of sunhemp infected with *Common bean mosaic virus*. *Egyptian J. Bot.* 21: 113-119.

Singh, R. and Singh, A. K. 1984. Effect of *Southern bean mosaic virus* infection on the leaf protein concentration in cowpea cultivars. *Curr. Sci.* 53: 390.

Sinha, A. and Srivastava, M. 2010. Biochemical changes in mungbean plants infected by *Mungbean yellow mosaic virus*. *Int. J. Virol.* 6: 150-157.

So, I. Y. 1980. Studies on ginger mosaic virus. *Korean J. Pl. Protec.* 19(2): 67-72.

Sohal, B. S. and Bajaj, K. L. 1993. Effects of *Yellow mosaic virus* on polyphenol metabolism in resistant and susceptible mung bean (*Vigna radiata* L. Wilczek) leaves. *Biochem. Physiol. Pflanz.*, 188: 419-423.

Sreelekha, L. 1987. Properties, host range and control of cowpea mosaic virus. M. Sc. Thesis, Kerala Agricultural University, Thrissur, 121p.

Srivastava, S. K. 1987. Peroxidase and polyphenol oxidase in *Brassica juncea* plants infected with *Macrophomina phaseolina* (Tassi) Goid and their implification in disease resistance. *Phytopathol.* 120: 249-254.

Sutha, R., Ramiah, M. and Rajappan, K. 1998 a. *Tomato spotted wilt virus* induced alterations in carbohydrate, syen and starch contents of tomato plants. *Int. J. Tropic. Pl. Dis.* 16: 247-252.

Sutha, R., Ramiah, M. and Rajappan, K. 1998 b. Effect of *Tomato spotted wilt virus* infection on the quality of tomato fruits. *Int. J. Tropic. Pl. Dis.*, 16: 261-265.

Taiwo, M. A., Kareem, K. T., Nsa, I. Y. and Hughes, J. D. A. 2007. Cowpea viruses: Effect of single and mixed infections on symptomatology and virus concentration. *Virol. J.* 4:95.

Thind, S. K., Monga, P. K., Kaur, N. and Cheema, S. S. 1996. Analysis of some biochemical and micronutrient constituents of *Yellow mosaic virus* infected mung. *Indian J. Virol.* 12: 157-159.

Thomas, J. E. 1986. Purification and properties of ginger chlorotic fleck virus. Ann. Appl. Biol., 108: 43-50.

Tsai, W. S., Shih, S. L., Kenyon, L., Green, S. K. and Jan, F. J. 2011. Temporal distribution and pathogenecity of the predominant tomato- infecting begomoviruses in Taiwan.*Pl. Pathol.* 60: 787-799.

Usharani, K. S., Surendranath, B., Khurana, S. M. P., Garg, I. D. and Malathi, V. G. 2004. Potato leaf curl- a new disease of potato in northern India caused by a strain of *Tomato leaf curl New Delhi virus*. *Pl. Pathol.* 53: 235.

van Loon, L. C., Bakker, P.A.H.M. and Pieterse, C. M. J. 1998. Systemic resistance induced by rhizosphere bacteria. *A. Rev. Phytopath.,* 36: 453-483.

Vasanthi, V. J. and Shanmugam, V. 2003. Physiological changes in cassava (*Manihot esculenta* Crantz) infected with Indian mosaic geminivirus. *Pl. Dis. Res.* 18: 196-198.

Veena, I. V. 2007. Induction of resistance against *Cowpea aphid borne mosaic virus* in *Vigna unguiculata* var. *sesquipedalis* (L.) Verdcourt. M. Sc. (Ag.) Thesis, Kerala Agricultural University, Thrissur. 93 p.

Verma, H. N. and Kumar, V. 1980. Prevention of plant virus disease by *Mirabilis jalapa* leaf extract. New Botanist., 7: 87-91.

Verma, H. N. and Prasad, V. 1984. Systemic induced resistance by plant extracts alters physiology of susceptible test host. *Indian J. Pl. Path.,* 5: 69-72.

Verma, H. N., Srivastava, S., Varsha and Kumar, D. 1986. Induction of systemic resistance in plant viruses by a basic protein from *Clerodendron aculeatum* leaves. *Phytopathol.* 89: 485-492.

Verma, V. S. 1974. Chemical compounds from *Azadirachta* as inhibitors of *Potato virus X*. *Acta. Microbiol. Polonica.,* 6: 9-13.

Vivanco, J. M., Querci, M. and Salazar, L. F. 1999. Antiviral and antiviroid activity of MAP containing extracts from *Mirabilis jalapa* roots. *Pl. Dis.,* 83: 1116-1121.

Wagih, E. E. and Coutts, R. H. A. 1982. Peroxidase, polyphenol oxidase and ribonuclease in tobacco necrosis virus infected or mannitol osmotically stressed cowpea and cucumber tissues. *Phytopath. Z.*, 104: 124-137.

Wei, G., Kloepper, J. W. and Tuzun, S. 1996. Induced systemic resistance to cucumber diseases and increased plant growth by plant growth-promoting rhizobacteria under field conditions. *Phytopath.,* 86: 221-224.

White, R. F. 1979. Acetyl salicylic acid (aspirin) induces resistance in tobacco. *Virol.*, 99: 410-412.

White, R. F., Duman, E., Shaw, P. and Antoniw, J. F. 1986. The chemical induction of PR(b) proteins and resistance to TMV infection in tobacco. *Antiviral. Res.*, 6: 177-185.

Wong, C. E., Carson, R. A. J. and Carr, J. P. 2002. Chemically induced virus resistance in *Arabidopsis thaliana* is independent of pathogenesis-related protein expression and the NPR 1 gene. *Mol. Pl. Microb. Interact.,* 15: 75-81.

Wyatt, S. D. and Brown, J. K. 1996. Detection of subgroup III geminivirus isolates in leaf extracts by degenerate primers and polymerase chain reaction. *Phytopathol.* 86: 1288–1293.

Yan, S. L., Lehrer, A. T., Hajirezaei, M. R. and Komar, E. 2008. Modulation of carbohydrate metabolism and chloroplast structure in sugarcane leaves which were infected by *Sugarcane yellow leaf virus* (SCYLV). *Physiol. Mol. Pl. Path.* 73: 78-87.

Yardimci, N., Eryigit, H. and Erdal, I. 2006. Effect of *Alfalfa mosaic virus* on the content of some macro and micronutrients in Alfalfa. *J. Cult. Collection.* 5: 90-93.

Zaidi, S. N. H., Singh, N., Ram, R., Zad, A. A. and Mukherjee, D. 1992. Changes in phenolic content and phenylalanine ammonia lyase in response to infection by carnation etch ring virus. *Indian J. Pl. Path.*, 10: 21-24.

Zaidi, Z. B., Gupta, V. P., Samad, A. and Naqui, Q. A. 1988. Inhibition of spinach mosaic virus by extracts of some medicinal plants. *Curr. Sci.,* 57: 151-153.

Zehnder, G. W., Yao, C., Murphy, J. F., Sikora, E. R. and Kloepper, J. W. 2000. Induction of resistance in tomato against cucumber mosaic cucumovirus by plant growth-promoting rhizobacteria. *Biol. Control.,* 45: 127-137.

Zhang, J., Borth, W. B., Lin, B., Dey, K. K., Melzer, M. J., Shen, H., Pu, X., Sun, D. and Hu, J. S. 2016. Deep sequencing of *Banana bract mosaic virus* from flowering ginger (*Alpinia purpurata*) and development of an immunocapture RT-LAMP detection assay. *Arch. Virol.,* 161: 1783-1795.

Zheng, L., Wayper, P. J., Gibbs, A. J., Fourment, M., Rodoni, B. C. and Gibbs, M. J. 2008. Accumulating variation at conserved sites in potyvirus genomes is driven by species discovery and affects degenerate primer design. *PLoS ONE 3*, e1586.

Abstract

Etiology and management of mosaic disease in ginger
(*Zingiber officinale* Roscoe)

by

ANANTHU. N

(2015 - 11 - 094)

THESIS

Submitted in partial fulfillment of the

requirements for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF PLANT PATHOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM-695522

KERALA, INDIA

2018

ABSTRACT

The study entitled 'Etiology and management of mosaic disease in ginger (*Zingiber officinale* Roscoe)' was conducted at the Department of Plant Pathology, College of Agriculture, Vellayani during 2015-2018 with the objectives to identify, characterize and sequence the genes of *Ginger mosaic virus* infecting ginger along with the management of the disease. As part of the study, the symptoms produced by the virus in ginger plants collected from the field and grown in glass house were observed. The symptoms on the leaves appeared as small light green flecks. These flecks eventually increased in size and formed streaks. The streaks were arranged parallel to the veins. The appearance of too many streaks on the leaves led to severe chlorosis and the leaves showed necrotic symptoms in the advanced stage.

Transmission of the virus was tested in rhizome (seed material collected from infected plants) and mechanical inoculation was done using infected leaf sap to the healthy plants. The infected rhizomes resulted in 100% transmission and mechanical transmission failed to transmit the virus.

Changes in total carbohydrates, chlorophyll, phenol, total soluble proteins and defense related enzymes namely peroxidase, polyphenol oxidase and phenylalanine ammonialyase were carried out at 30, 60, 90 and 120 days after infection. The study revealed an increase in the content of phenols and defense related enzymes in infected plants. An increase in carbohydrates, chlorophyll and protein in healthy plants was also observed. Protein profile study using sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS- PAGE) indicated the presence of a novel protein with molecular weight of 20 kDa in the infected plant sample.

The immunological detection techniques direct antigen coating- enzyme linked immunosorbant assay (DAC- ELISA) and dot immunobinding assay (DIBA) were carried out. Since the etiology of the virus was unknown, seven suspected viruses were tested in DAC-ELISA and antibodies specific to two viruses namely *Banana bract mosaic virus* (BBrMV), *Cucumber mosaic virus* (CMV) gave an absorbance value of 0.15 and 0.19 respectively which was three times more than the absorbance shown by the healthy leaf (0.05 and 0.07). The infected leaf tested for the presence of *African cassava mosaic virus* (ACMV) by triple antibody sandwich ELISA (TAS-ELISA) gave an absorbance of 0.0425 while the healthy gave an absorbance of 0.017. DIBA analysis gave positive reaction to BBrMV.

Polymerase chain reaction (PCR) was carried out with both total DNA and RNA isolated from infected ginger leaf sample. The PCR experiment was positive to Begomoviruses and negative to PVY (*Potato virus Y*) and BBrMV isolates. An amplicon of size 550 bp was obtained for the sample DNA using begomo degenerate primer. The sequence was subjected to BLAST analysis which indicated 74 per cent similarity to *Tomato leaf curl virus Bangalore isolate.*

The management studies were conducted with antiviral principles like botanicals, chemicals and that of microbial origin against the virus. The experiment was conducted in completely randomized design (CRD) with 13 treatments and three replications. Karthika was the variety used for the study. Perfekt (a botanical extract-76%) at 0.5 ml L^{-1} and 1ml L^{-1}, chemicals namely aspirin, salicylic acid, barium chloride, at 100 and 150 ppm concentrations and botanicals namely ten per cent leaf extracts of *Mirabilis jalapa* and *Bougainvillea spectabilis*, two per cent neem oil garlic emulsion and two per cent PGPR mix II were used in the experiment. The treatments were given at fortnightly interval. Before each spray the efficacy of the treatments were evaluated using Vulnerability Index (V.I) developed by Bos (1982). The treatments with Perfekt at the rate of 0.5 ml L^{-1} and 1.0 ml L^{-1} and ten per cent leaf extract of *Mirabilis jalapa* were found effective for the management of the disease.

The study indicates that the mosaic disease in ginger is transmitted through rhizomes, the virus has been molecularly characterized and shows only 74% identity with *Tomato leaf curl virus* (TLCV) and management of the disease can be done by application of Perfekt at 0.5 ml L^{-1} or 10% leaf extract of *Mirabilis jalapa* at fortnightly interval.

Appendices

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 II

ESTIMATION OF PROTEIN

1. 0.1 M Sodium acetate buffer (pH 4.7)

Stock solutions

A: 0.2 M solution of Acetic acid (11.5 ml in 1000 ml)

B: 0.2 M solution of Sodium acetate (16.4 g of $C_2H_3O_2Na$ or 27.2 g of $C_2H_3O_2Na.3H_2O$ 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 wasdissolved 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°C. The working dye was prepared just before use by diluting the stock solution to five times with water.

APPENDIX III

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 upto 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
TrisHCl (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

TrisHCl (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 4^0C for up to 2 weeks.

6. **Ammonium persulphate solution**

Ammonium persulphate-0.1g

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 PCR Analysis

1. 50X 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 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

5m*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

Bromochloroindolyl 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 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 X

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 XI

BUFFERS FOR PCR PRODUCT

50 x TAE for running the gel

Tris Base - 242g

Glacial Acetic acid -57.1 ml

0.5 M EDTA (pH 8.0) - 100ml

Make the volume to 1 l

1.2% agarose gel is prepared for casting and examination of PCR product

APPENDIX XI

PCR Product Sequence of Begomovirus isolate

**>Ginger.begomo_AV.forward_6874-1_P0941,Raw Sequence(602 bp)CATTAAGATAGGCAGATGCTATGATAGTAGCCAGATGAAATGATGTA
AGTCAGATCCATCATTGTGGGGCAGAAGATATGTATGGAGAAAAATGC
AACCTAGGGAACCTTAAGAAGCGCATTTACGTTTTGTGGAAATGTCGT
TCCTGTTAGGAAAAGTTTGGTTCTTGATAGGACAAGACTGGACCTACA
CTAAGAGTCTCATAGTTTCCTTGCCCGCAACCGATGCGTGCGATAGCC
CACAAGAGTGTGGAGATGTGTTCGATATAGAGGAAAAATGAGTTTAGC
ACCGCACCGGCAAGAATAGGAGCGAGATTGAAATCAGGGGCTAAGGC
CATTTCATGAACTGCCACTGGGGGCATCACGCAGCAATGAGGCCGCG
TTAGTTAAAAGTGGGGAAGATTCTCAATCAGGAAGTTACAACCCGCGG
ATTGGGGGAACATGACCACCATAGGATTTTCTTTGCGTGTCTTGTCTG
TACCCATGCTCCAACTAATGGGGGAGGGGTTAAAGCCCTTTTTGTTGT
TTTTCCGTCCTAAGGCGGACCCGCGATTCACGAGAATGGGGGATTACT
TAAAAAAGTTTTTGCTGGAGCAGGAGCCT**

174319