Varietal screening of black pepper to *Cucumber mosaic virus* and *Piper yellow mottle virus* and their sero-molecular detection

by

ARYA M.

(2018-11-013)

THESIS

Submitted in partial fulfilment 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

2020

DECLARATION

I, hereby declare that this thesis entitled **"Varietal screening of black pepper to *Cucumber mosaic virus* and *Piper yellow mottle virus* and their sero-molecular detection"** 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, associate ship, fellowship or other similar title, of any other University or Society.

Vellayani **Arya M.**

Date: (2018-11-013)

CERTIFICATE

Certified that this thesis entitled **"Varietal screening of black pepper to *Cucumber mosaic virus* and *Piper yellow mottle virus* and their sero-molecular detection"** is a record of research work done independently by Ms. Arya M. under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associate ship to her.

Vellayani
Date:

Dr. Radhika N. S.
Assistant Professor
Department of Plant Pathology
College of Agriculture, Vellayani
(Chairperson)

CERTIFICATE

We, the undersigned members of the advisory committee of Ms. Arya M. a candidate for the degree of **Master of Science in Agriculture** with major in Plant Pathology, agree that the thesis entitled **"Varietal screening of black pepper to *Cucumber mosaic virus* and *Piper yellow mottle virus* and their sero-molecular detection"** may be submitted by Ms. Arya M., in partial fulfillment of the requirement for the degree.

Dr. Radhika N. S.
Assistant Professor
Department of Plant pathology
College of Agriculture, Vellayani
(Chairperson)

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

Dr. Heera G.
Assistant Professor
Department of Plant Pathology
College of Agriculture, Vellayani
(Member)

Dr. Beena R.
Assistant professor (Plant physiology)
Department of Plant physiology
College of Agriculture, Vellayani
(Member)

ACKNOWLEDGEMENT

I bow my head before the almighty for the blessings throughout this journey in completion of my work. I am expressing my sincere gratitude to my esteemed chairman ***Dr. Radhika N. S****, Assistant Professor, Department of Plant Pathology, College of Agriculture, Vellayani. This work would not have been possible without her guidance, constant support and encouragement.*

I would like to thank ***Dr. Joy M****., Associate Professor and Head, Department Plant Pathology and all the members of my thesis advisory committee* ***Dr. Heera G****. Department of Plant Pathology and* ***Dr. Beena R****. Department of Plant Physiology. Their insightful comments and constructive criticisms at different stages of my research were thought-provoking and they helped me focus my ideas.*

My sincere thanks to ***Dr. Umamaheswaran K.*** *(Rtd. Professor, Department of Plant Pathology) for unfailing patience, moral support and encouragement he bestowed upon me during the course of study.*

I would like to thank ***Dr. Ayisha R****., Department of Plant Pathology for the immense support and encouragement which was a great blessing for me to overcome difficult times. I wish to acknowledge all the teaching, nonteaching staff and students of Department of Plant Pathology for their assistance and help.*

I gratefully acknowledge Kerala Agricultural University and institutes which helped during my research work, viz; Cardamom research station, Pampadumpara; Pepper Research Station Panniyur, RARS Ambalavayal and IISR Farm, Peruvannamuzhi. I am grateful to the scientists ***Dr. Simi, Dr. Dhanya, Dr.Yamini****, Farm officer* ***Hareesh sir*** *working in these institutes for giving the opportunity to work and availing important plant materials and suggestions.*

My sincere gratitude to ***Bincy chechi, Bhavana chechi, Deepti chechi and Deepa chechi*** *for their support. I am lucky enough to have the support of many good friends like* ***Deena, Chippy, Athira, Veny, Tejasree, Haritha, Aswathy, Divya, Anit, Sinija chechi and my dear juniors***.

I would also like to thank my parents ***Rajendran D.*** *and* ***Meera Devi*** *and other family members for their immense support.*

Any omission in this brief acknowledgement does not mean lack of gratitude.

Arya M.

CONTENTS

LIST OF TABLES

13	Reaction of samples collected from Karunapuram panchayat to antibodies of CMV and BSV in DAS-ELISA	
14	Reaction of samples collected from Ambalavayal panchayat to antibodies of CMV and BSV in DAS-ELISA	
15	Reaction of samples collected from Meenangadi panchayat to antibodies of CMV and BSV in DAS-ELISA	
16	Reaction of samples collected from Poothadi panchayat to antibodies of CMV and BSV in DAS-ELISA	
17	Reaction of samples collected from Panamaram panchayat to antibodies of CMV and BSV in DAS-ELISA	
18	Reaction of stunted disease infected black pepper plants to respective polyclonal antibodies of CMV and SCBV	
19	Reaction of stunted disease infected black pepper plants to respective polyclonal antibodies of CMV and SCBV	
20	Consolidated results of ELISA and DIBA conducted for the samples collected from Idukki and Wayanad district	
21	Quality and quantity of DNA isolated from infected leaf samples in Idukki district recorded using spectrophotometer	
22	Quality and quantity of DNA isolated from infected leaf samples in Wayanad district recorded using spectrophotometer	
23	NCBI BLAST analysis for sequences similarity of PYMoV isolate from survey locations in Idukki with other PYMoV isolates reported in black pepper.	
24	NCBI BLAST analysis for sequences similarity of PYMoV isolate from survey locations in Wayanad with other PYMoV isolates reported in black pepper.	

25	NCBI BLAST analysis for sequences similarity of PYMoV isolate from Erattiyar, Idukki with other badnavirus isolates	
26	NCBI BLAST analysis for sequences similarity of PYMoV isolate from Kattappana, Idukki with other badnavirus isolates	
27	NCBI BLAST analysis for sequences similarity of PYMoV isolate from Pampadumpara, Idukki with other badnavirus isolates	
28	NCBI BLAST analysis for sequences similarity of PYMoV isolate from Karunapuram, Idukki with other badnavirus isolates	
29	NCBI BLAST analysis for sequences similarity of PYMoV isolate from Ambalavayal, Wayanad with other badnavirus isolates	
30	NCBI BLAST analysis for sequences similarity of PYMoV isolate from Meenangadi, Wayanad with other badnavirus isolates	
31	NCBI BLAST analysis for sequences similarity of PYMoV isolate from Poothadi, Wayanad with other badnavirus isolates	
32	NCBI BLAST analysis for sequences similarity of PYMoV isolate from Panamaram, Wayanad with other badnavirus isolates	

LIST OF FIGURES

LIST OF PLATES

17	Variety Karimunda infected with stunted disease	
18	Variety Panniyur 1 infected with stunted disease	
19	Variety Thekkan pepper infected with stunted disease	
20	Screening of varieties for reaction to viruses	
21	Symptom observed on grafted Karimunda plant 40 DAG	
22	Symptom observed on grafted Thevam plant 58 DAG	
23	Symptom observed on grafted Girimunda plant 75 DAG	
24	Colour development in ELISA plate by interaction of virus infected samples with their respective antibodies	
25	DIBA conducted for detection of CMV and PYMoV infected samples	
26	Symptoms due to single infection of either CMV or PYMoV	
27	Electrophoresis gel image of amplified DNA of PYMoV infected samples from Idukki and Wayanad	
28	Phylogeny tree showing the virus isolates in three different clades	

LIST OF APPENDICES

LIST OF ABBREVIATIONS

µl	Micro litre
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumin
BSOLV	*Banana streak obino l'Ewai virus*
BSMYV	*Banana streak mysore virus*
CMV	*Cucumber mosaic cucumovirus*
CP	Coat protein
DAS-ELISA	Double Antibody Sandwich -Enzyme linked immunosorbent assay
DAG	Days after grafting
DIBA	Dot immunq binding assay
DIECA	Diethyl dithiocarbamate
DNA	Deoxy Ribonucleic acid
H	Hour
Ha	Hectare
kbp	Kilo base
kDa	Kilo dalton
Kg	Kilo gram
l	Litre
M	Molar
MAb	Monoclonal antibody
mg	milligram
min	minute
mL	millilitre
Mm	millimolar

MW	Molecular weight
NCM	Nitro cellulose membrane
NCBI	National Centre for Biotechnology Information
Nm	Nano meter
OD	Optical density
ORF	Open reading frame
PAb	Polyclonal antibody
PAL	Phenylalanine ammonia lyase
PBS-TPO	Phosphate Buffer Saline -Tween Polyvinyl pyrrolidone ovalbumin
PCR	Polymerase chain reaction
PO	Peroxidase
PPO	Polyphenol oxidase
PVP	Poly vinyl pyrrolidone
PVY	*Potato virus Y*
PYMoV	*Piper yellow mottle virus*
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RT-PCR	Reverse transcription-Polymerase chain reaction
SDM	Spray dried milk
TBS	Tris buffer saline
TbRSV	*Tobacco ringspot virus*
TmRSV	*Tomato ringspot virus*
TMV	*Tobacco mosaic virus*
ZYMV	*Zucchini yellow mosaic virus*

Introduction

1. INTRODUCTION

Black pepper known as “King of Spices” and “Black Gold” is one of the most valuable and earliest known spices in the world. Black pepper (*Piper nigrum* L.), which originated in the tropical evergreen forests of the Western Ghats in India has captured global attention and ardor that widened over the years. Leading black pepper producing countries in the world are India, Indonesia, Malaysia, Sri Lanka, Thailand, Vietnam, Brazil and China (NHB, 2016). The course of Indian history have been variously influenced by black pepper trade. The cropped area under black pepper is around 4.76 lakh ha with 4.13 lakhs tons production in the world during 2016. India is having largest area under black pepper (1.83 lakh ha) followed by Indonesia (1.43 lakh ha) (NHB, 2016)

Black pepper cultivation in India is mainly spread across Kerala, Karnataka, Tamil Nadu, Goa, Pondicherry and North Eastern states. Karnataka contributes about 27 per cent of area and 54.6 per cent of total production of black pepper with a productivity of 471 kg ha^{-1}. While Kerala having 63 per cent of cultivated area contributes 34.3 per cent of the production is having a productivity of 254 kg ha^{-1} (Senthilkumar and Swarupa, 2017). Most of the black pepper farms in Kerala are small or medium holdings ($\leq$ 0.2 ha). Other reasons regarding low productivity are cultivation of poor yielding vines, senile and unproductive vines, lack of quality planting materials, losses due to pests, diseases and drought, and price fluctuations (Senthilkumar and Swarupa, 2017).

Viral diseases are the third among the serious diseases of black pepper after foot rot and anthracnose. Since black pepper is a vegetatively propagated crop, chances of spreading virus among crop is high. Sarma *et al.* (2001) reported the occurrence of *Cucumber mosaic virus* (CMV) infecting black pepper in India. The symptoms caused by CMV are small, crinkled, brittle and leathery leaves; and chlorotic patches or streaks

on the leaves. Purification and partial characterization of the virus was done and concluded it as belonging to the sub group IB.

Bhat *et al.* (2005a) reported the presence of a badnavirus associated with stunted disease of black pepper which is transmitted by mealy bug. The disease is caused due to any of these viruses or due to mixed infections. Many of the virus infected plants remain symptomless during certain climatic conditions hence external symptoms cannot be a good criteria for the identification of the disease. Yield loss varying from 16 to 85 per cent was observed in virus infected plants (IISR, 2005). Vertical transmission along with the symptomless vines will accelerate the spread of the disease. Along with these, some insect vectors and weed plants are also contributing to spread of the disease. Severity of the disease was found more in higher altitudes and in poorly managed crop fields.

The primary step in controlling the disease should focus on the production of disease free planting materials. Regular monitoring and rouging of the infected plants along with potential weed hosts in the field and nursery becomes a necessity. Hence proper awareness needs to be given among farmer regarding the incidence, spread and need of its proper management. Development of rapid, sensitive and reliable techniques for detecting both symptomatic and symptomless sources needs to be given special attention which leads way to the development of a certified planting material production system in case of black pepper.

The present study was undertaken with the objectives like survey and collection of viruses infected black pepper plants from Idukki and Wayanad districts, symptomatology studies, screening of selected varieties of black pepper for resistance against viruses, serological diagnosis and molecular characterization of the viruses.

Review of literature

2. REVIEW OF LITERATURE

Black pepper (*Piper nigrum* L.) is one among the spice crop cultivated in Kerala having high export potential and attained global interest. One of the major yield limiting factors in black pepper is the incidence of viral diseases. The outline of technical programme includes.

- Survey and collection of virus infected black pepper plants
- Symptomatology
- Screening of selected varieties of black pepper
- Serological diagnosis and molecular characterization of viruses

2.1 SURVEY AND COLLECTION OF VIRUS INFECTED BLACK PEPPER PLANTS

Occurrence of viruses similar to disorders has been reported around the world in different black pepper growing countries. First report on the occurrence of a virus disease in black pepper was from Indochina (Bharat, 1952) and Malaysia (Holliday, 1959). Virus disease infecting black pepper became more predominant in Sri Lanka and was widely seen as small leaves on the infected vines (Alles, 1984). Hence it was termed as "little leaf disease of black pepper". Similar diseases have been reported throughout the black pepper growing countries in South - East Asia, Africa and Latin America (Costa *et al*., 1969; Alles, 1984).

Virus disease of black pepper was reported in India from a black pepper nursery at Neriamangalam in Idukki District of Kerala during 1975 (Paily *et al*., 1981) and in Pulpally of Wayanad District of Kerala in 1978 (Sarma *et al*., 1992). Later it was named as Piper yellow mottle disease caused by *Piper yellow mottle virus* (PYMV) by Lockhart in 1993. The disease was given a uniform term as 'Stunted Disease of Black Pepper' at the 1991 International Workshop on Black Pepper Diseases held at Lampung, Indonesia (Wahid 1992).

Further studies done at Indian Institute of Spice Research (IISR), Kozhikode, Kerala revealed the presence of two viruses *viz. Cucumber mosaic virus* belonging to genus cucumovirus and *Piper yellow mottle virus* belonging to genus badnavirus (Sarma *et al*., 2001; Bhat *et al*., 2004) albeit the disease is caused by either of these viruses or due to the association of both viruses. The mottle disease incidence reached 95 per cent in Bangka and Lampung (Indonesia) in 2005 (Lakani, 2006). High incidence of the disease was also observed in Yogyakarta (86 – 93.75 per cent) (Alif, 2018).

Disease incidence in Lampung (Indonesia) increased from 23.3 per cent in 1987 to 40.0 per cent in 1990 (Firdausil *et al*, 1992). Bhat *et al*. (2005b) observed a high disease incidence and severity in black pepper grown in high altitudes. Highest disease incidence was found in Wayanad (45.4 per cent) and Idukki (29.4 per cent) districts of Kerala. Similar studies conducted in Kodagu district of Karnataka recorded 7-100 percentage disease incidence (Bhat *et al*., 2005b). Parthasarathy *et al*. (2005) observed that as the altitude varied between 750–800 m in Wayanad and between 900-1500 m in Idukki, the disease incidence also varied between 45 per cent and 90 per cent respectively. Yield loss varying from 16 to 85 per cent was observed in the virus infected plants (IISR, 2005).

Ayisha (2010) conducted a survey in the black pepper growing tracts of Thiruvananthapuram and Kollam districts and found that the virus disease is wide spread in these two districts. Around 50 per cent of the survey plots were reported to have a disease incidence of 1-25 percentage whereas 30 per cent of the plots showed 26-50 percentage disease incidence and only 5 per cent showed severe disease incidence (51-75 percentage). Umadevi *et al*. (2016) reported an increased severity of the disease under abiotic stresses and high temperature.

Miftakhurohmah *et al*. (2020) conducted a survey on the incidence and severity of mottle disease of black pepper plants in Sukamulya Research Station, Sukabumi

Regency, West Java and reported the disease incidence of 100 per cent and severity of 32.50 per cent.

Presence of vectors

The primary spread of the viruses occurs through infected planting materials while the secondary spread is by means of various insect vectors. Bharat (1952) and Holliday (1959) transmitted a possible virus disease from infected black pepper using the mealy bug *Planococcus citri*. The disease transmission experiments conducted by De Silva (1996) showed that PYMoV is transmitted by mealy bugs and lace bugs; and also through the infected rooted cuttings. Transmission experiments results confirmed mealy bug, *Planococcus citri* (Risso) and pepper lace bug, *Diconocoris distanti* (Drakes) as vectors of PYMoV. Disease transmission from the infected to healthy black pepper was successful when the first nymphal stage of the mealy bugs was used as vector. Fleck necrosis, reduction of leaf size and increasing of leaf thickness were observed on the test plants used for lace bug transmission. This was the first report of *D. distanti* as a virus vector and a vector of PYMoV.

Lockhart *et al.* (1997) reported that Thailand isolates of PYMoV from both *P. nigrum* and *P. betle* were transmitted by *P. citri* from naturally infected *P. nigrum* and *P. betle*, to healthy seedlings of *P. nigrum* cv. Krabi. It was found that on inoculation by viruliferous mealybugs, test plants developed vein-clearing and chlorotic mottle symptoms after 5–8 weeks. In Brazil, PYMoV was suspected to be transmitted by another species of mealybug, *Pseudococcus elisae* (Durate *et al*, 2001).

Bhat *et al.* (2004) reported the transmission of virus from diseased to healthy black pepper plants cv. Karimunda by grafting and mealybug, *Ferrisia virgata*. The initial symptoms were vein clearing and chlorotic mottle, and observed in 14 of 20 test plants at five weeks after inoculation. The CMV isolate was transmitted to indicator plants by mechanical inoculation and by the vector *Aphis gossypii*, but not by *Myzus*

persicae; nevertheless neither mechanical nor insect transmission of CMV to black pepper was successful (Bhat *et al.*, 2005c).

Ayisha (2010) studied the insect transmission using aphids and mealy bugs. Per cent transmission using aphids *Toxoptera aurantii* was 10 and chlorotic spots developed at two weeks after inoculation. Disease transmission was found successful using *F. virgata* producing the initial symptoms as chlorotic mottling after two months of inoculation with 30 per cent transmission rate. *Phenococus solenopsis* failed to transmit the virus.

2.2 SYMPTOMATOLOGY

Caner and Ikeda (1972) reported a viral disease in Brazil with chlorotic, irregular yellowish areas among green areas of *Piper nigrum.* Paily *et al.* (1981) reported a serious malformation on black pepper with the infected leaves became mottled, narrow and crinkled. Randombage and Bandra (1984) observed little leaf disease of black pepper in SriLanka. The important symptoms recorded were chlorosis of leaves, shortening of internodes, proliferation of branches, greening and enlargement of floral bracts. Little leaf infected vines had reduced leaf size, the number of flowers per spike and berry weight. Germination of seeds obtained from infected vines were comparatively less compared to that obtained from healthy ones. The disease has been described as little leaf in Sri Lanka (Randombage and Bandra, 1984), mosaic disease in India (Prakasham *et al.*, 1990) wrinkled leaf disease in Malaysia (Kueh and Sim, 1992) and as stunted disease in Indonesia (Sitepu and Kasim, 1991; Firdausil *et al*, 1992).

Kueh and Sim (1992) differentiated the symptoms into four categories *viz.*, stunting, reduction in size of internodes, narrowing of leaves, and marginal necrosis and chlorosis. The diseased plants showed reduction in height, girth of column, internode length and leaf area (Eng *et al.*, 1993).

Lockhart *et al*. (1997) identified PYMoV, a mealy bug transmitted badnavirus infecting *Piper spp*. Field symptoms observed were, mild to severe chlorotic mottling, vein-clearing, interveinal chlorosis, reduction in leaf size, leaf puckering and deformation from Sarawak (Malaysia), Sri Lanka, Philippines and Thailand. Chlorotic mottle symptoms without leaf deformation were also observed on betelvine in Thailand.

Non-enveloped bacilliform virus-like particles resembling those of badnaviruses were observed through electron microscope (EM) in partially purified extracts of symptomatic black pepper and betelvine plants (Lockhart and Olszewski, 1994). They named the disease as *Piper yellow mottle*, and described the nature, mode of transmission and distribution of the causal agent, which was named *Piper yellow mottle virus* (PYMoV). Stunted disease caused by was characterized by chlorotic mottling, vein clearing, leaf distortions, reduced plant vigour, chlorosis and poor fruit set (Durate *et al*., 2001).

Sarma *et al*. (2001) reported the partial characterization of a virus associated with stunt disease of black pepper in South India and identified as an isolate of CMV. Symptoms like interveinal yellow flecking, yellow mottling, dark-green vein banding, narrow distorted leathery leaves and overall reduction in the growth of the infected black pepper in the Wayanad district of Kerala were similar to symptoms described on black pepper in several South-East Asian countries.

Previous reports from South-East Asian countries indicate that stunted disease of black pepper with varied foliar symptoms is a disease complex caused by more than one type of virus. The etiology of the disease was uncertain and it was found that symptoms of stunted disease were caused by complex of viruses (a badnavirus, a spherical virus and a clostero-like virus particle) (Eng *et al*., 1993). Symptoms of yellow mottle disease contained a mixture of viruses in Sri Lankan black pepper: PYMoV (30 × 130 nm), CMV, 30 nm diameter isometric, and unidentified isometric

virus-like particles (30 nm diameter) (De Silva, 2002). Infection by PYMoV is characterized by vein clearing, scattered chlorotic flecks followed by chlorotic mottling along veins leading to interveinal chlorosis and characteristic curling of the leaves. In addition, vein banding, vein thickening and green island-like symptoms were also noticed. The infected vines had reduced vigour and yield (Bhat *et al.*, 2005b).

Ayisha (2010) observed small chlorotic spots on young leaves which later become small, sickle shaped, leathery and stunted. Vein clearing, mottling, curling and twisting of leaves were also observed. However, the infected plants need not produce visible symptoms under certain environmental conditions. But the virus could be transmitted from these symptomless sources through mealy bug and by grafting. These symptomless sources can act as a means of secondary spread in the field (Bhat *et al.*, 2012). Occurrence of endogenous PYMoV in black pepper was reported in which ORF 3 was found to be frequently integrated (Bhat *et al.*, 2017).

Herbers *et al*. (1997) found that symptoms like chlorosis, stunting and mosaic was associated with the accumulation of starch and sugars. Umadevi *et al*. (2016) studied the effect of temperature, changes in host factors associated with symptom expression. PCR tested negative plants showed the presence of virus in low copy number when subjected to temperature stress. The amount of total chlorophyll and total phenols were detected higher in healthy plants compared to the infected plants. But after symptom expression the total sugars and total proteins were found higher. In healthy plants the IAA content was same before and after stress. But in the infected plants, the IAA content was higher after stress. Role of phenolics, IAA, total sugars and chlorophyll were studied in symptom expression and reported that there was an increase in phenolics, IAA and total sugars and decrease in chlorophyll. The presence of virus encoded proteins (photosystem I protein RNAse H protein of BSV,

Chaperonin 60 -2 protein ORF III polyprotein) along with host proteins were also detected in black pepper plants after exposure to temperature stress.

Two viruses namely, *Cucumber mosaic virus* and *Piper yellow mottle virus* are reported to be associated with this disease. Costa *et al.* (1969) reported that following inoculation with *Cucumber mosaic virus*, symptoms of stunted disease was reproduced in black pepper. Partial characterization and identification of a virus associated with stunted disease in South India revealed it as an isolate of CMV. The leaf extract of diseased black pepper or virus purified from diseased black pepper leaves in direct antigen coating-ELISA and electroblot immunoassay tests reacted positively with polyclonal antisera of cucumber mosaic virus (CMV)-Banana (India), CMV-Brinjal (India), CMV-Chilli (India), CMV-Tomato (India), CMV-L (USA) and CMV-A (China) (Sarma *et al*., 2001).

CMV infecting black pepper was reported to be transmitted mechanically on to *Nicotiana benthamiana* and *N. glutinosa* in Sri Lanka (De Silva *et al*., 2001). The CMV infecting black pepper was mechanically transmitted and propagated in the tobacco species, *N. benthamiana* and *N. glutinosa*. Symptoms appeared within 7-10 days of inoculation. Symptoms on *N. benthamiana* included severe puckering, mosaic, mottling and downward curling of leaves while in N. *glutinosa,* vein clearing followed by mosaic and yellow mottling with slight curling at leaf margins were found. In other naturally infected *Piper* species such as *P. chaba* Hunter, *P. colubrinum* Link and *P. longum* L, infection by CMV was detected. Mosaic and stunting symptoms were found on infected *P. chaba* and *P. longum* whereas no such symptoms were seen on *P. colubrinum* infected with CMV. Thus *P. colubrinum* might act as an asymptomatic carrier for CMV. It was seen that out of 79 *Piper coloubrinum* tested, 13 were found to contain the infection of CMV. CMV infection was detected on host species like *Ageratum conyzoides, Colocasia eseulanta, Synedrella nodiflora, Cynodon daetylon*

and *Sonehus oleraeeus* in DAS-ELISA. In *Glyricidia sepium*, one commonly used standards in black pepper plantations, CMV infection was detected (Bhat *et al*. 2004).

CMV belonging to genus Cucumovirus, family Bromoviridae, is a single stranded RNA virus with isometric particles (Palukaitis *et al*., 1992). The coat protein gene of an isolate of CMV was cloned and sequenced. CMV coat protein gene was amplified using RT-PCR from black pepper samples collected from Belur. A PCR product of expected size (650 bp) was observed in all the infected samples. It was found that the coat protein gene consists of 657 nucleotides and it encodes a protein of 218 amino acids. The gene was closely related to the CMV affecting Egyptian henbane plant in India, which is a member of CMV subgroup I. Sequence analysis showed 92-97 percentage amino acid identity with members of sub group I and 77-79 percentage with members of subgroup II. It was concluded that CMV infecting black pepper in India is a member of CMV sub group I. Bhat *et al*. (2017) has undertaken the complete genome sequencing of CMV from black pepper and it revealed a rare deletion in the methyl transferase domain of 1a gene. Complete nucleotide sequence of the CMV-black pepper isolate comprised RNA1 (3349 NT), RNA2 (3049 NT) and RNA3 (2217 NT). RNA1 has a single ORF coding for 990 amino acids 1a protein. RNA2 and RNA3 each have two ORFs. ORF 3a codes for the movement protein with 279 amino acids and ORF 3b codes for 218 amino acids long coat protein.

PYMoV is a circular non-covalently closed double stranded DNA virus belonging to genus *Badnavirus*, family *Caulimoviridae* having bacilliform particle morphology.

Ayisha (2010) observed chlorotic spots on the seedlings germinated by planting seeds from infected plants on three months after germination with 26 per cent transmission rate. Presence of both viruses was found on cotyledons and seed coat while CMV was detected on embryo only. Deeshma and Bhat (2014) experimentally proved the seed transmission of PYMoV where DNA was isolated from anther,

endosperm, embryo and perisperm and subjected to PCR using specific PYMoV primers. Presence of virus was detected in all these parts and also in the anthers of variety Panniyur 1 indicating a possibility for pollen transmission. True seed transmission of PYMoV was confirmed by the presence of virus in the embryo and in the seedlings raised by germinating these seeds.

Siju and Bhat (2008) studied a virus isolate affecting *Piper betle* and *Piper longum*. *Piper betle* exhibited mottling, mosaic and reduction in leaf size while *P. longum* showed dark green patches, blisters, mosaic and distortion in leaf size as the major symptoms. A PCR product of around 600 bp was obtained from the ORF III region of the *Badnavirus* infecting these hosts. On cloning and sequencing, highest sequence identity (>89.1% at nucleotide level and >93.4% at amino acid level) was shown with PYMoV from India infecting black pepper. On phylogenetic analysis, it was found that isolates of *P. betle and P. longum* clustered together with PYMoV isolates and *P. betle* isolate was found closer to PYMoV black pepper isolate than *P. longum* isolate. Thus, it was concluded that, *Badnavirus* infecting *P. betle* and *P. longum* in India is a strain of PYMoV.

Hany *et al.* (2014) studied the complete genome sequence of PYMoV and the genome which is 7.2 kb consists of four open reading frames (ORFs) with ORF 1, 2 and 4 having molecular mass of 15.7, 17.1 and 17.9 kDa. ORF3 of PYMoV is a polyprotein of 218.6 kDa which encodes a viral movement protein (MP), trimeric dUTPase, zinc finger, retropepsin, RT-LTR, and RNAse H.

2.3 SCREENING FOR RESISTANCE

Identifying the sources of resistance is an important measure for proper management of disease. Bhat *et al*. (2005b) indexed a total of 2186 black pepper nursery plants from eleven varieties by DAS-ELISA for the presence of viruses. Of the 714 plants tested positive, 390 plants exhibited no visible external symptoms indicating the

necessity for sensitive diagnosis in identifying virus-free planting material. IISR-Sreekara had the highest number of infected plants followed by IISR-Subhakara while Panniyur-5 had the least number of infected plants.

Bhat (2008) proposed PCR based method for indexing 845 plants representing 14 popular varieties of black pepper which were asymptomatic for PYMoV. It was found that 694 plants were positive (82 per cent) for PYMoV. The percentage of infected plants ranged from 59 to 100 among different varieties. 100 per cent infection was noticed in Panniyur-6 and Sreekara had the least number of infected plants (59 per cent). Some of the indexed PCR positive plants exhibited visible symptoms such as mild chlorosis, yellow specks and mottling in 1-3 months. The study showed that indexing by PCR successfully detected PYMoV infected black pepper plants showing masking of symptoms. This method can be used in certification programmes in order to identify disease free plants.

Ayisha (2010) conducted varietal screening where, twelve varieties were tested for their resistance to viruses and the disease assessment was done based on vulnerability index on 0-5 scale. It was found that Panniyur 2, 3 and 4 were moderately resistant whereas moderate susceptibility was recorded for Panniyur 5, 6, 7, Malabar Excel and Shakthi. Kuthiravallypadappan and Kottanadan were found to be susceptible while Karimunda was highly susceptible.

2.4 SEROLOGICAL AND MOLECULAR CHARACTERIZATION OF VIRUSES

2.4.1 Enzyme linked immunosorbent assay (ELISA)

The infected samples reacted positively in indirect antigen coating-ELISA and electro blot immunoassay tests with polyclonal antisera of CMV-Banana (India), CMV-Brinjal (India), CMV-Chilli (India), CMV-Tomato (India), CMV-L (USA) and

CMV-A (China) (Sarma, 2001). ELISA with PYMoV always gave low absorbance values, and did not separate PYMoV-infected and healthy plants successfully (De Silva *et al*., 2002). Bhat *et al*. (2004) standardized a Double Antibody Sandwich-Enzyme Linked Immunosorbent Assay (DAS-ELISA) method for detection of CMV in infected black pepper samples. Varied distribution of CMV was there within diseased plant samples although the virus was found in all plant parts including stems and roots. Detection of CMV in black pepper samples was successful using heterologous polyclonal antisera raised against different strains of CMV such as CMV-A, CMV-B, CMV-C, CMVL,CMV-Fiji, CMV-Passiflora and CMV-T while few antisera failed to detect the virus. It was the first report regarding the production of polyclonal antisera for the detection of CMV infecting black pepper.

Bhadramurthy *et al*. (2005) reported a standard DAS-ELISA technique for the detection of a badnavirus in black pepper. It was possible to identify infected samples from the healthy samples with an increase in the optical density (OD) values. However, some of the samples exhibiting symptoms of the disease gave OD values close to that of the healthy samples due to low titre value.

Masking of symptoms was a major constraint in identifying healthy and infected plants. Remission of symptoms on black pepper plants infected with stunted disease during monsoon season makes it difficult to identify infected plants; hence sensitive diagnosis is necessary. When 2186 black pepper nursery plants were indexed by DAS-ELISA for CMV and PYMoV, 714 vines were found infected with at least one of the two viruses. When DAS-ELISA positive vines were checked for the presence of any external visible symptoms, 390 plants out of 714 showed no visible external symptoms. Subsequently, the variation in the concentration of CMV and PYMoV was found during different months of a year. Higher titre was observed for both viruses from October to February. Higher absorbance values were recorded for Panniyur 1 followed by Panchami whereas lowest absorbance was recorded in IISR

Malabar Excel throughout the year compared to other varieties. Stable titre value for both CMV and PYMoV were recorded in variety Karimunda. No measurable concentration of virus was detected in IISR Malabar Excel in the month of March. During October to November, higher concentration of virus was detected in root, stem, and berries of Panniyur 1 while in remaining months roots contained less amount of both viruses. When the concentration of virus in different parts of the plant was tested, the presence of PYMoV was detected in both older and younger leaves. In case of CMV, higher concentration was found in younger leaf. (Bhadramurthy *et al*., 2008). This result indicated that a plant cannot be considered as healthy merely based on visual symptoms. Ayisha (2010) used polyclonal antibodies of CMV, BSV (Banana) and SCBV (Sugarcane) to detect the viruses by ELISA. Out of 12 samples, six samples were positive for both CMV and badna virus which shows a mixed infection of both the viruses.

2.4.2 Dot Immunobinding assay (DIBA)

Polyclonal antibodies of CMV and BSV were used for the detection of the viruses by DIBA and the analyzed samples showed positive reaction towards CMV and BSV (Ayisha, 2010).

2.4.3 Molecular diagnosis

The pattern of electrophoretic migration of nucleic acid obtained from a Thailand isolate of PYMoV was found similar to ComYMV (*Commelina yellow mottle virus*) but differences in the number and band positions were observed. PYMoV nucleic acid was resistant to RNAse but DNase could completely degrade them. ComYMV when digested with S1 nuclease (endonuclease for hydrolyzing single stranded DNA or RNA), two smaller genomic fragments were obtained due to cleavage at the two discontinuities in the circular genome (Medberry *et al.,* 1990). PYMoV when treated with S1 nuclease, no change in electrophoretic pattern was noticed. It was assumed that this may be due

to one or more genomic discontinuities in PYMoV as described for other pararetroviruses.

Lockhart *et al*. (1997) made use of PCR to amplify genomic PYMoV sequences using badnavirus-specific oligonucleotide primers, and sequence analysis comparisons of the putative reverse transcriptase (RT) domain showed PYMoV ORF III to be closely related to other mealybug-transmitted badna viruses like BSV, CoYMV and SCBV than to RTBV, a leafhopper-transmitted badnavirus.

De Silva *et al*. (2002) amplified the genomic DNA obtained from infected black pepper samples using different oligonucleotide primer combinations. DNA bands of 700 bp size got amplified from infected black pepper extracts. PYMoV DNA was only amplified by the primer pair combination Badna-T and SCBV-R1.

Siju *et al*. (2008) amplified PCR product of approximately 600 bp from the ORF III region of the *Badnavirus* infecting betel vine and Indian long pepper and confirmed PCR as an efficient method for the detection of PYMoV different piper species. A protocol for total DNA isolation from black pepper was standardized (De Silva *et al.*, 2002; Hareesh and Bhat, 2008). These studies showed that PCR method can be successfully used for the detection of PYMoV infection in black pepper especially in plants showing masking of symptoms. Siju *et al*. (2008) reported a sensitive method for total RNA isolation using sodium sulphite at 0.5 per cent. By performing RT-PCR, coat protein gene of *Cucumber mosaic virus* was amplified and a product of expected size (~650 bp) was observed in the infected plants.

Bhat and Siju (2007) developed a single-tube multiplex RT–PCR for the simultaneous detection of *Cucumber mosaic virus* and *Piper yellow mottle virus* in black pepper. Protocol for the isolation of total RNA was further modified to isolate DNA at the interphase stage. Both DNA and RNA were allowed to co-precipitate in a single tube at the isopropanol stage. Multiplex PCR (m-PCR) is a type of PCR in which two or more

loci are amplified simultaneously in a single reaction using several pairs of primers. PCR primers designed for amplifying 650 bp from the coat protein gene of CMV and 450 bp from the open reading frame I of PYMoV were used in the reaction. The method is rapid, reliable and aids in the rapid screening of a large number of plants for both viruses.

Molecular diagnosis was performed using PCR for identification of the causal viruses. PCR product of 600bp amplicon size was obtained when primer specific for coat protein gene of CMV infecting banana was used (Ayisha, 2010).

Bhat and Siju (2014) standardized SYBR green-based real-time PCR and real-time RT-PCR assays for the detection of PYMoV and CMV, respectively in black pepper. The positive samples showed a melting peak at 82.5-84°C. Field samples when analyzed using this method distinguished more number of infected plants than conventional PCR. This method has been found to be up to 1000 times more sensitive than conventional PCR and RT-PCR and a good method for screening mother plants.

2.4.4 Quantity and quality of DNA

Lalwan *et al*. (2014) observed that spectrophotometric analysis is the most commonly used method for assessment of quality and quantity of nucleic acid. Absorbance ratio at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of about 1.8 is accepted as pure for DNA.

2.4.5 Sequencing and blast analysis

Hany *et al*. (2014) reported the first complete genome sequence of *Piper yellow mottle virus* and confirmed that it is a member of the family *Caulimoviridae* genus *badnavirus*. ORF1 of PYMoV has 45 per cent similarity to the *Fig badna virus* ORF1, 42-44 per cent identity was observed with different isolates of *Citrus yellow mosaic virus* (CYMV) (AC055656) and *Cacao swollen shoot virus* (CSSV). ORF2 of PYMoV showed around 44 per cent identity to CYMV (NP-569152) and 39 per cent identity

to *Dioscorea bacilliform virus* (DBV). (AB147985.1) while ORF3 was having 50 per cent similarity to ORF3 of DBV (YP-001036293) and 55 per cent identity to CYMV (NP-569153). ORF 4 of PYMoV was found to have 78 per cent similarity with a hypothetical protein whose sequence has been given as a partial genome sequence of PYMoV (ABI30246).

Bhat *et al.* (2014) studied the sequence diversity among badnavirus isolates and related species in India. The study showed the presence of PYMoV in eight different species of Piper. Sequence variability in the conserved RT/RNAse H region of virus was studied using badnavirus specific primers in different isolates collected from cultivars of black pepper. One isolate from different species of *Piper* was also used for the analysis. It was found that four species did not produce the expected amplicon size while four other species showed similarity to sequences of plants. Remaining isolates along with, *P. attenuatum, P. betle, P. galeatum, P. argyrophyllum P. longum, , P. barberi P. sarmentosumand, P. ornatum, P. colubrinum P. trichostachyon* showed a high identity of 90 per cent at the amino acid level and 85 per cent at the nucleotide level indicating that they are isolates of PYMoV. Among isolates infecting *P. peepuloides, P. bababudani P. mullesua P. chaba*, and *P. thomsonii*, high sequence variability was found at both nucleic acid level and amino acid level. On phylogenetic analysis, it was found that all PYMoV isolates clustered together and separated from other badnaviruses. The criteria for defining species in badnaviruses are a) 80 per cent identity (in the nucleotide sequence) or b) 89 per cent identity (in the amino acid sequence) in the RT/RNase H region. The BLAST analysis results from *P. peepuloides, P. mullesua* and *P. thomsonii* showed 68 per cent identity with different badnaviruses such as, *Taro bacilliform virus (*TBV), *Musa acuminata endogenous badnavirus* and *Canna streak virus* respectively. This suspects the occurrence of new badnaviruses among these species.

Similar kind of sequence variability was reported among isolates of another badnavirus CYMV infecting different citrus species such as *C. jambhiri, C. grandis, C. aurantifolia and C. sinensis* (Borah *et al*. 2009; Johnson *et al*. 2012).

2.4.6 Construction of phylogeny tree

Molecular Evolutionary Genetics Analysis (MEGA) software is developed for the analysis of DNA and protein sequences on a comparative basis and aids in studying the evolutionary patterns of genes, genomes and species on molecular level (Kumar *et al*., 1994; Tamura *et al*., 2014). Large number of sequences consisting of contemporary data sets can be used in Rel Time method. For determining the ordering and spacing of sequence divergence events in species and gene family, relative time estimates produced by MEGA will be useful. MEGA software helps in exploring the evolutionary relationships of homologous sequences and in estimating the selective and neutral divergence of evolutionary relationship among sequences.

Phylogenetic analysis showed that PYMoV is closely related to *Cacao swollen shoot virus* (CSSV), *Citrus yellow mosaic virus* (CYMoV) and *Dioscorea bacilliform virus* (DBV) (Hany *et al*., 2013). Miftakhurohmah *et al*. (2020) studied the amino acid and nucleotide similarity of PYMoV isolate in Sukamulya and found a high similarity with PYMoV isolates from India and China, i.e. 89.7 – 93.5 percentage and 90.2 – 93.9 percentage respectively. Phylogenetic analysis showed that PYMoV isolate from Sukamulya is clustered with PYMoV isolates from India. A close phylogenetic relationship between PYMoV isolate from Sukamulya and from India indicates that both viruses share a similar origin, maybe migrated to Indonesia through infected plant materials or insect vectors.

Materials and methods

3. MATERIALS AND METHODS

The present study entitled "Varietal screening of black pepper to *Cucumber mosaic virus* and *Piper yellow mottle virus* and their sero-molecular detection" was conducted at Department of Plant Pathology, College of Agriculture Vellayani during the year 2018-2020. The following procedures were used for the objectives mentioned in the study.

3.1 SURVEY AND COLLECTION OF VIRUS INFECTED BLACK PEPPER PLANTS

Survey was conducted in the major black pepper growing tracts of Kerala, Wayanad and Idukki (Plate 1). From each districts two taluks were selected; from each taluk, two panchayats were selected; and five farmers were selected from each panchayats (Plate 2, 3). Disease incidence was noted from farmer's field. A survey proforma was prepared with the details about cultivar/variety, age of the crop, standards used.

3.1.1 Disease incidence (DI), Vulnerability Index (VI) and Presence of vectors

Disease incidence (DI)

The per cent disease incidence was estimated by calculating the number of infected plants out of the total plants observed in the field.

$$DI = \frac{\text{Number of infected plants X 100}}{\text{Total number of plants observed}}$$

Vulnerability index (VI)

0 - No symptoms

1 - Light mottling of younger leaves

2 - Mottling of leaves and younger leaves

3 - Leaf curling and distortion of leaves

4 - Distortion and reduction of leaf size

5 - stunting of plant

$$V.I. = \frac{(0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4 + 5n_5) \times 100}{n_t(n_c - 1)}$$ Bos (1982)

n0, n1……n5 = Number of plants in category 0, 1, 2, 3, 4 and 5

nt = total number of plants

nc= total number of categories

Presence of vectors

From the plots surveyed, presence of vectors was examined.

3.1.2 Maintenance of inoculum

The healthy and disease infected plants were maintained at College of Agriculture, Vellayani under insect proof greenhouse conditions.

3.2 SYMPTOMATOLOGY

The different types of symptoms observed at the farmer field were collected and recorded. Specimens showing diverse symptoms were taken for serological and molecular diagnosis.

3.3 SCREENING FOR DISEASE RESISTANCE

Rooted cuttings of different black pepper varieties were raised in pots and grafted with infected scion. These grafted plants were observed for the

expression of symptoms. The nature of symptom expression and time taken for the expression of symptoms were recorded. The expressed symptoms were recorded using a disease score scale of 0-5 (Plate 4) and calculation of vulnerability index was done using the equation of Bos (1982). Grading of black pepper varieties on the basis of vulnerability index was done (Table 1)

$$V.I. = \frac{(0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4 + 5n_5) \times 100}{n_t (n_c - 1)}$$

Table 1. Grading and reaction of black pepper varieties on the basis of vulnerability index

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

3.4 SEROLOGICAL AND MOLECULAR CHARACTERIZATION OF VIRUSES

3.4.1 DAS-ELISA

Samples collected from farmer's field were subjected to DAS-ELISA using the procedure described below (Huguenot *et al.*, 1993).

In DAS-ELISA, the antigen is coated between two layers of antibody. Primary antibodies of CMV and BSV (DSMZ- Germany) at the dilution of 1:1000 and 1: 200 respectively were initially added to ELISA plates with coating buffer. Incubation was done for 2h at 37°C. The wells are then washed with phosphate buffer saline tween (PBS-T) for three times at 3 minutes interval using ELISA plate washer (pw-40, BIORAD). Homogenization of one gram of infected and healthy leaf samples in 5 ml of coating buffer (carbonate buffer) which contains 2 per cent (w/v) polyvinyl pyrrolidone (PVP) was done. The homogenate was transferred to an eppendorf tube and centrifuged at 5000 rpm for 10 min at 4°C (Hetich Zentrifugen). 200 µl of antigen was dispensed in to Tarson immunological plates, incubated for 1 h at 37°C in an incubator and washed with PBS-T. Blocking was done using 5 per cent spray dried milk (SDM) 200µl to cover the unbound surfaces and incubated the plate for 1 h at 37°C. Primary antibodies diluted in PBS-TPO were added (200µl) and incubated at 37°C for 2h. Secondary antibody conjugate at 1:10,000 dilutions were added to the plates; incubated for 2 h at 37°C and washed with PBS-T using ELISA plate washer. The substrate para nitro phenyl phosphate (pNPP) in substrate buffer (1 mg per 1ml) was added to each well (200 µl per well). Incubation was done for 5-10 minutes at room temperature. Reaction was stopped by adding 50 µl of 4 per cent sodium hydroxide.

Absorbance was read using an ELISA reader at 405 nm (Microplate Reader 680, BIORAD). Absorbance value of samples when found two times or more when compared to the OD value of healthy sample was considered as positive.

3.4.2 Dot immuno binding assay (DIBA) for the detection of virus infected black pepper (Banttari and Goodwin, 1985)

Leaf tissues of the infected or healthy plant samples were homogenized using antigen extraction buffer (1:5 w/v) and filtered through a cheese cloth or cotton. In an eppendorf tube 0.8 ml of the extracted sap was taken to which 0.4 ml of chloroform was added. The mixture was vortexed well and centrifuged at

12,000 g for two minutes. Nitrocellulose membrane (NCM) in 1x1 cm dimension was cut and floated on tris buffer saline (TBS) and air dried. Spot the sample (5-10 µl) to the centre of each square and allowed to dry. NCM spotted with samples were immersed in blocking solution (TBS-SDM) with slight oscillation for one hour at room temperature. NCM was treated with TBS for 10 minutes and incubated in crude antiserum diluted at 1:1000 either overnight at 4°C or at room temperature for 2 hours**.** NCM was again washed in TBS for 10 minutes and incubated in secondary antibody (antirabbit IgG alkaline phosphate conjugate diluted at 1: 10,000 in TBS- SDM) for 1 hour at room temperature. NCM was again rinsed in TBS for 10 minutes and incubated in a solution of nitro blue tetrazolium salt (NBT) (0.33 mg/ml of substrate buffer) and bromo chloro indoyl phosphate (BCIP) (0.175 mg/ml of substrate buffer) in the room temperature at dark for colour development. NCM was rinsed in fixing solution for 10 minutes at room temperature. After colour development and air dried between Whatmann No1 filter paper, the colour development was analyzed in Gel Doc system (Gel DocTM XR+). The leaves of apparently healthy plants were used as control.

3.4.3 Molecular characterization

Isolation of DNA (Doyle and Doyle, 1990)

Two hundred mg of plant tissue was taken and ground it to fine powder using liquid nitrogen or keep in deep freezer for one hour prior to grinding. CTAB buffer is preheated to 65°C and 2ml was added and homogenized. One ml of the extract was transferred to each 2ml eppendorf tubes. The samples were incubated in a water bath at 65°C for 30 minutes with occasional mixing. After incubation, the samples were centrifuged at 10000 rpm for 10 minutes. Later, the supernatant was transferred to clean microfuge tubes. 500µl of Chloroform: Iso amyl alcohol (24:1) was added to each tube and mixed by inversion in a zig zag motion. Again the tubes were spinned at 10000 rpm for 10minutes. The upper aqueous phase containing DNA was

transferred to a clean microfuge tube. DNA was precipitated using ice cold isopropanol. Equal volume of ice cold isopropanol or absolute ethanol was added to each tube and mixed well. The mixture was incubated at 4°C for overnight to precipitate the DNA. The DNA was precipitated as a pellet by spinning the tube at 10000 rpm for 7 minutes. The supernatant was discarded and DNA pellet was washed using ice cold 70 per cent ethanol to remove salt contamination. The pellet was spinned down at 10000 rpm for 5 minutes. The resultant pellet was air dried for 15 minutes and dissolved in 50µl of TE (Tris EDTA buffer) buffer.

Agarose gel electrophoresis

The presence of total DNA can be confirmed by Agarose gel electrophoresis. Agarose powder 0.6 g was added to 50 ml of 1X TAE buffer. The solution was heated in a microwave for the complete melting of agarose. After cooling, 1.8 µl of ethidium bromide was added and then poured to the horizontal gel electrophoresis unit (Hoefer Power Pack, Germany). 1kb ladder (GeNei) was used as molecular marker. Eight µl of DNA was mixed with 2 µl of 6X loading dye and dispensed into the wells in the gel. The gel was run at 65V for 2 h in TAE buffer. Gel was removed when the dye reached three fourth distance of gel. DNA was visualized using UV transilluminator system (Bio-Rad) and documented in Gel Doc system (Gel Doc TM XR+)

3.4.4 Quality of DNA obtained

Quality of DNA was tested by reading the absorbance value using bio-spectrophotometer. 5 µl of DNA was dissolved in 1400 µl of TE buffer taken in cuvette and TE buffer alone was taken as blank. Using a bio-spectrophotometer, absorbance readings were taken at 260 and 280 nm wavelengths. A260/A280

gives the value of quality of DNA obtained. Good quality DNA will have a 260/280 value round 1.8-2.0.

Quantity of DNA

Concentration (µg/ml) = A260 X dilution factor X 50µg ml^{-1}

1 OD 260 Unit= 50 µg ml^{-1} for dsDNA

3.4.5 Sequencing and Blast analysis

Table 2. Primers specific to ORF III region of the virus used for the amplification of *Piper yellow mottle virus* (PYMoV)

Primer	**Sequence (5`-3`)**	**Product size (bp)**	**Reference**
PYMoV - F PYMoV - R	CTATATGAATGGCTAGTGATG TTCCTAGGTTTGGTATGTATG	400	Bhatt and Siju (2009)

Table 3. Polymerase Chain Reaction (PCR) conditions set for the amplification of ORF III of PYMoV

Event	Temperature	Minutes	Cycles
Initial denaturation	94°C	3	1
Denaturation	94°C	0.5	34
Annealing	56°C	1	
Extension	72°C	1	
Final extension	72°C	10	1

Table 4. Preparation of master mix

Master mix components	Quantity (25 µl)
Water	17.2 µl
PCR buffer 10X	2.5 µl
$MgCl_2$ 25mM	2.5 µl
DNTPs 10mM	0.5 µl
Forward primer 10µM	0.5 µl
Reverse primer10µM	0.5 µl
Taq DNA polymerase 5units/ µl	0.3 µl
DNA sample	1 µl

Agarose Gel electrophoresis for PCR products

PCR was done using primers specific to ORF III of PYMoV (Table 2). The temperature profile for PCR and preparation of master mix are detailed in (Table 3and 4) The PCR products were checked by running in 1.2% agarose gel electrophoresis. The gel was prepared by adding agarose powder 0.6 g to 50 ml of 1X TAE buffer. The solution is heated in a microwave for the complete melting of agarose. After cooling, 1.8 µl of ethidium bromide was added and poured to the horizontal gel electrophoresis unit (Hoefer Power Pack, Germany). 1kb ladder (GeNei) was used as molecular marker. Five µl of DNA was mixed with 2 µl of 6X loading dye and dispensed into the wells in the gel. The gel was run at 65V for 2 h in TAE buffer. Gel was removed when the dye reached three fourth distance of gel. DNA was visualized using UV transilluminator system (Bio-Rad) and documented in Gel Doc system (Gel Doc TM XR+). The amplified products were given for sequencing at Agri genome Pvt. Ltd., Kochi.

Sequencing was done for the amplified PCR products and was subjected to BLAST analysis to study sequence similarity of the isolated samples with already reported sequences in NCBI on National Centre for Biotechnological Information (NCBI) site.

3.4.6. Construction of phylogeny tree

Phylogeny tree was constructed using MEGA 6 software. Homologous series showing similarity to isolated samples were aligned to construct the phylogeny tree.

Results

4. RESULTS

Incidence of viral disease is one among the several yield limiting factors in black pepper. Hence, it is important to study the distribution of the viruses, characterize them and screen the varieties for disease resistance. With these objectives the present study was undertaken at College of Agriculture, Vellayani during the period from 2018-2020. The results of the study are as follows.

4.1 SURVEY AND COLLECTION OF VIRUS INFECTED BLACK PEPPER PLANTS

4.1.1 Disease incidence, vulnerability index and presence of vectors

Survey for the viral diseases of black pepper was conducted from May 2019 to February 2020 in Idukki and Wayanad districts of Kerala. Two panchayats were selected from two blocks in each districts; and five farmers were selected from each panchayats. From Idukki district, Kattappana and Nedumkandam blocks were selected. The four panchayats were Erattiyar, Kattappana, Pampadumpara and Karunapuram. From Wayanad district, Panamaram and Bathery blocks were selected and the panchayats were Poothadi, Panamaram, Ambalavayal and Meenangadi. 40 farmers field were visited during the survey. The pepper varieties viz., Panniyur 1 and Karimunda were cultivated by most of the surveyed farmers.

Disease incidence was recorded from all panchayats in the selected blocks of Idukki and Wayanad district.

In Idukki district, highest disease incidence was noted in Erattiyar panchayat (37.40 per cent) and lowest in Karunapuram panchayat (21.62 %) whereas in Wayanad district highest incidence was recorded at Poothadi (45.50 %) and lowest in Meenangadi panchayat (24.50 %) (Table 5).

Table 5. Viral disease incidence (DI) in black pepper at different survey locations in Idukki and Wayanad districts

District	Panchayat	Disease incidence (%)
Idukki	Erattiyar	37.40
	Kattappana	32.40
	Pampadumpara	29.72
	Karunapuram	21.62
Wayanad	Ambalavayal	42.20
	Meenangadi	24.50
	Poothadi	45.50
	Panamaram	36.41

Table 6. Vulnerability index (VI) of viral diseases of black pepper at different survey locations in Idukki and Wayanad districts

Panchayat	Vulnerability index
Erattiyar	45 ± 0.577
Kattappana	41 ± 0.577
Pampadumpara	32 ± 0.231
Karunapuram	41 ± 1.764
Ambalavayal	37 ± 2.028
Meenangadi	38 ± 0.306
Poothadi	58 ± 0.577
Panamaram	61 ± 2.309

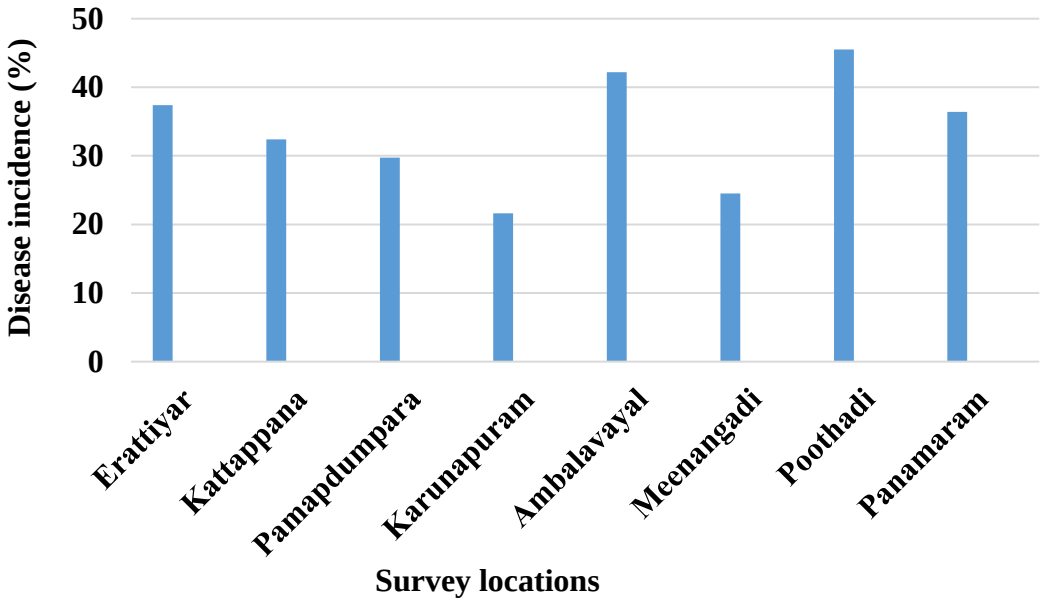


Fig. 1 Incidence of stunted disease recorded in different panchayats of Idukki and Wayanad districts.

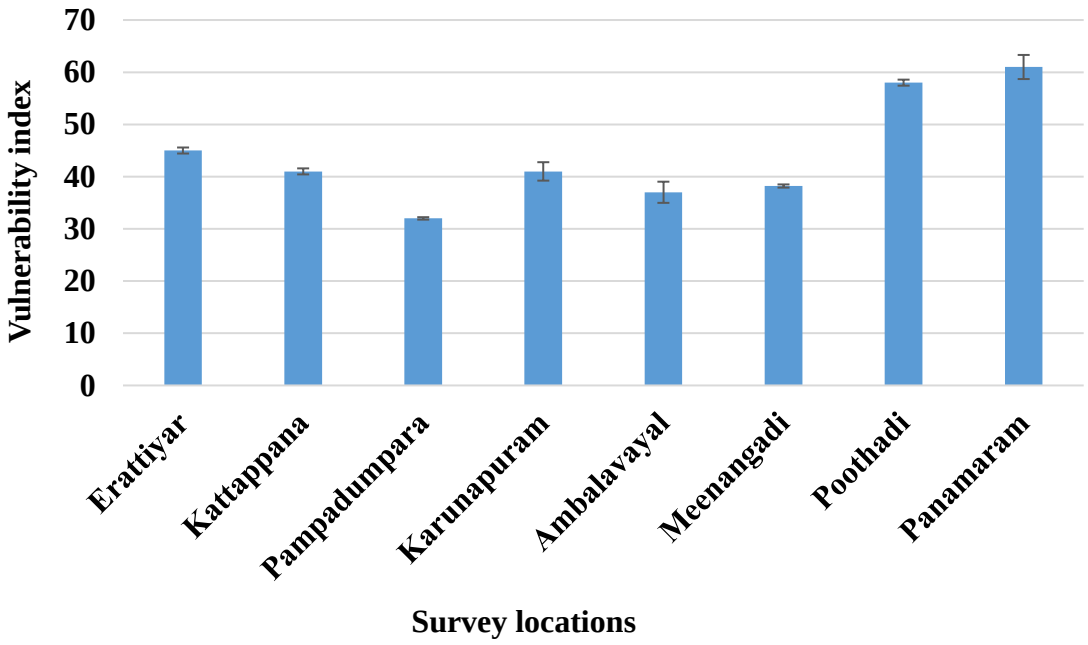


Fig. 2 Vulnerability index of stunted disease in black pepper recorded in different panchayats of Idukki and Wayanad districts

Intensity of infection was indicated by Vulnerability Index (VI). The VI for the selected plants in each area was calculated based on the disease score developed by Ayisha, (2010) which ranged from 0-5. In Idukki district, the vulnerability index recorded in Erattiyar, Kattappana, Pampadumpara, Karunapuram panchayat were 45, 41, 32 and 41 respectively. For Wayanad district, 37, 38, 58 and 61 were the vulnerability index recorded in Ambalavayal, Meenangadi, Poothadi and Panamaram panchayats respectively. Highest VI was recorded in Erattiyar panchayat (45) in Idukki district while lowest was recorded at Pampadumpara (32).In Wayanad VI was highest in Panamaram (61) and lowest in Ambalavayal (37) (Table 6).

Presence of mealybugs was observed on the lower surface of leaves and on the black pepper vines in the survey locations (Plate 5, 6).

4.1.2 Maintenance of the inoculum

Infected black pepper plants were collected from field, maintained in insect proof greenhouse conditions and observed for the expression of symptoms. Symptoms expression was more evident during warmer periods and gradually disappeared after a rain (Plate 7).

4.2 SYMPTOMATOLOGY

Symptoms observed in the infected samples collected from field were chlorotic spots, vein banding, distortion and reduction in leaf size, general chlorosis, puckering of leaves and vein clearing (Plate 8). Symptoms were generally observed on leaves. It was observed that symptoms were more severe in poorly managed fields without no proper fertilization and weeding. Symptoms initially appeared as small chlorotic spots gradually leading to inter venal chlorosis, vein clearing, severe mottling and vein banding. Reduction and distortion of leaf size and stunting of growth were also observed in the virus infected plants. Puckering of leaves, reduction in leaf size, mottling and chlorosis were observed in Erattiyar panchayat (Plate 9). In Kattappana panchayat, mottling, reduced leaf size and chlorotic specks were observed (Plate 10).

Plate 5. Presence of mealy bugs identified in the virus infected field

Plate 6. Stereo microscopic image of mealy bug at 50X

Plate 7. Virus infected black pepper plants maintained under insect proof greenhouse condition

a. Chlorosis

b. Chlorotic spots

c. Vein banding

d. Distortion and leaf size reduction

e. Puckering of leaves

f. Vein clearing

Plate 8 (a-f). Different symptoms of viral infection in black pepper observed in the field

a. Puckering of leaves

b. Reduction in leaf size

c. Mottling

d. Chlorosis

Plate 9 (a-d). Virus infected black pepper samples from Erattiyar, Idukki

a. Mottling **b. Reduction in leaf size**

c. Small chlorotic specks

Plate 10 (a-c). Virus infected black pepper samples from Kattappana, Idukki

a. Chlorotic spots

b. Reduction in leaf size

c. Chlorosis

d. Distortion of leaf

Plate 11 (a-d). Virus infected black pepper samples from Pampadumpara, Idukki

a. Chlorotic specks on leaves

b. Chlorotic spots

Plate 12 (a-b). Virus infected black pepper samples from Karunapuram, Idukki

a. Vein banding

b. Chlorotic spots

c. Mottling

Plate 13 (a-c). Virus infected black pepper samples from Ambalavayal, Wayanad

a. Distortion of leaf

b. Chlorosis

c. Reduction in leaf size

Plate 14 (a-c). Virus infected samples from Meenangadi, Wayanad

a. Severe mottling

b. Chlorosis

c. Reduction in leaf size

Plate 15 (a-c). Virus infected samples from Poothadi, Wayanad

a. Mottling

b. Vein clearing

Plate 16 (a-b). Virus infected samples from Panamaram, Wayanad

Chlorotic spots, reduction in leaf size, general chlorosis and distortions of leaves were observed in Pampadumpara panchayat (Plate 11). But in Karunapuram panchayat, small chlorotic specks were mainly observed (Plate 12). In Wayanad districts symptoms were observed in much severe form than in Idukki which is clear from the Vulnerability Index recorded. Vein banding, chlorotic spots and mottling were observed in Ambalavayal panchayat (Plate 13) while in Meenangadi panchayat distortion of leaf, chlorosis and reduction in leaf size were observed (Plate 14). Severe mottling along with chlorotic and distorted leaves was observed in Poothadi panchayat (Plate 15) whereas in Panamaram panchayat, mottling and vein clearing were noticed (Plate 16).

4.2.1 Disease incidence (DI) and Vulnerability index (VI)

Natural incidence of the disease as well as vulnerability index of the black pepper varieties in the surveyed plots were recorded (Table 7 and 8). Karimunda and Kottanadan were the local varieties cultivated by farmers in both Idukki and Wayanad districts. The incidence of the disease recorded was 70 per cent in Karimunda followed by 66 per cent in Kottanadan. Other cultivated varieties *viz*., Neelamundi and Shakthi recorded 50 per cent while Panniyur recorded 54 per cent. The VI was recorded based on the disease score scale 0-5. Vulnerability index of zero is considered as a resistant variety whereas VI in the range of 1 to 25 is considered as moderately resistant. Variety is categorized as moderately susceptible when VI of 26 to 40 is recorded and 41 to 50 recorded as susceptible. VI greater than 50 is considered as highly susceptible. It was found that Karimunda plants were severely affected by the disease followed by Kottanadan and Neelamundi. Karimunda and Kottanadan belonged to highly susceptible category with VI value 58 and 51 respectively (Plate 17). The disease incidences recorded were also high for both the varieties (70 and 66 per cent respectively). Neelamundi was found to be susceptible with a DI of 50 per cent and VI of 45. Shakti, Panniyur and Thekkan pepper belonged to moderately susceptible category with VI

values of 39, 36 and 34 respectively (Plate 18 and 19). No visible symptoms were observed on Thekkan pepper plants grafted on *Piper coloubrinum*.

Table 7. Viral disease incidence (DI) in different varieties of black pepper observed in the field

Varieties	DI (%)
Karimunda	70
Kottanadan	66
Neelamundi	50
Shakti	50
Panniyur	54
Thekkan pepper	40
Thekkan pepper grafted with *Piper coloubrinum*	0

Table 8. Vulnerability index (VI) of viral diseases of black pepper for different varieties in the field

Varieties	VI
Karimunda	58 ± 0.577
Kottanadan	52 ± 1.764
Neelamundi	45 ± 2.848
Shakti	38 ± 1.764
Panniyur	36 ± 0.577
Thekkan pepper	34 ± 0.664
Thekkan pepper grafted with *Piper coloubrinum*	0

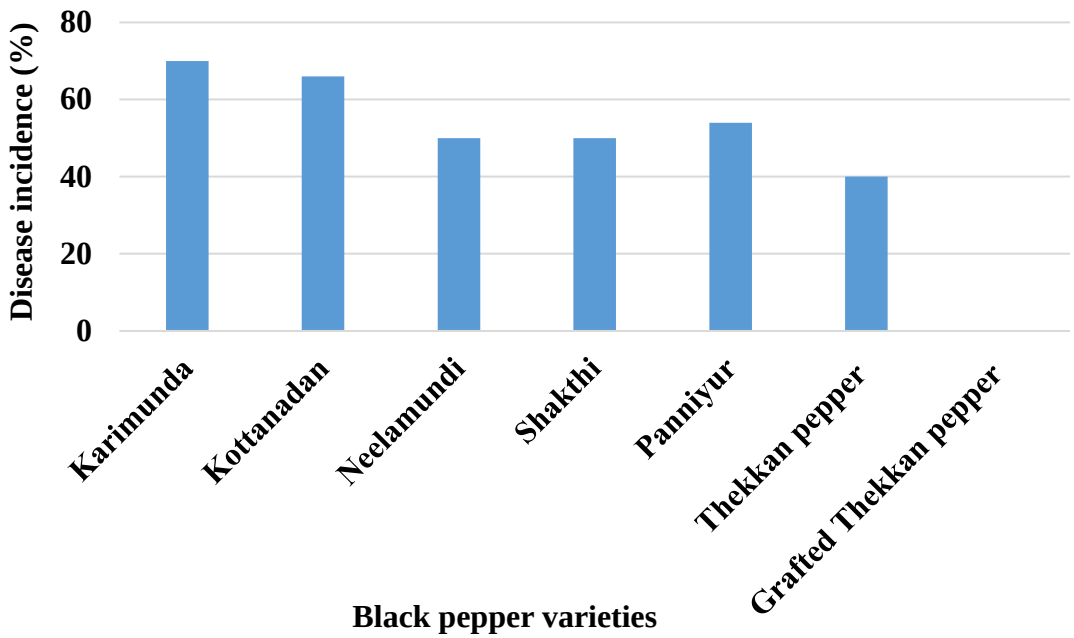


Fig. 3 Incidence of stunted disease observed in different varieties of black pepper

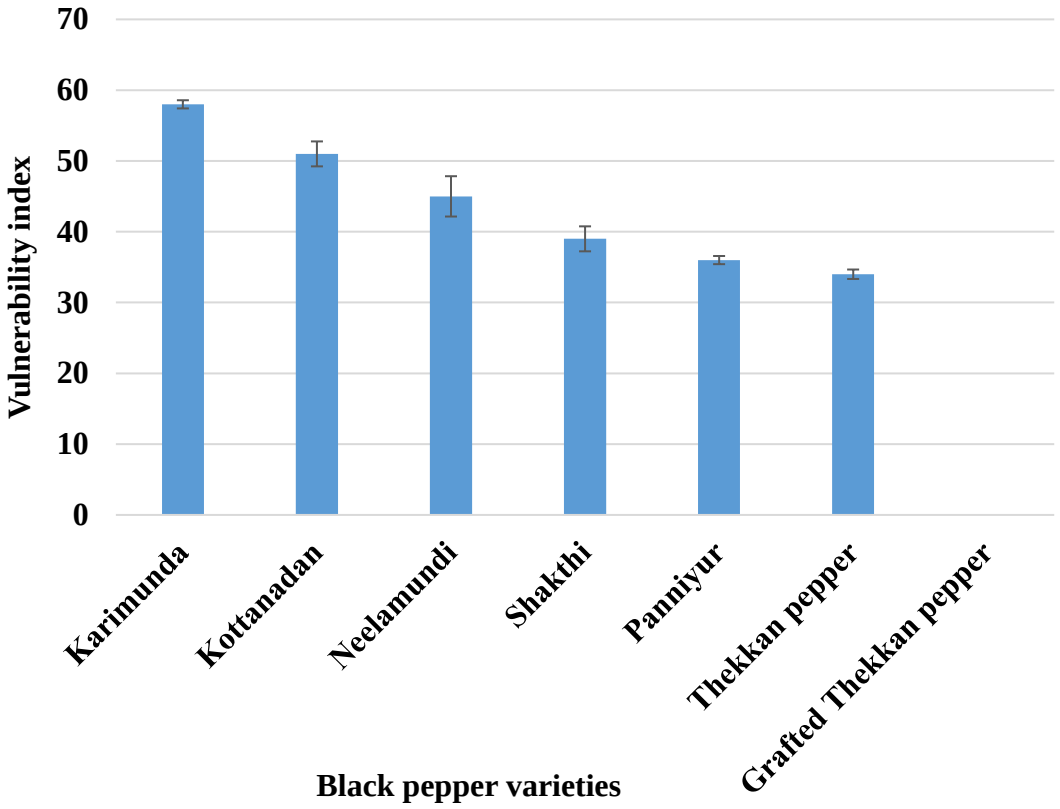


Fig. 4 Vulnerability index of stunted disease observed in different varieties of black pepper

Plate 17. Var. Karimunda plant infected with stunted disease

Plate 18. Var. Panniyur 1 infected with stunted disease

Plate 19. Var. Thekkan pepper infected with stunted disease

4.3 VARIETAL SCREENING

The varietal screening was done on the basis of days taken for symptom expression, nature of symptom development and vulnerability index (Table 9). The healthy plants of 15 varieties were grafted with infected scions of Karimunda and were maintained under insect proof conditions (Plate 20). Rooted cuttings of Panniyur varieties (1 to 8), IISR varieties like Sreekara, Subhakara, Thevam, Shakthi, Girimunda, Panchami and local cultivar Karimunda with three replications were maintained. Chlorotic spots were observed on grafted Karimunda plants on 40^{th} day after grafting and a vulnerability index of 48 was recorded (Plate 21). Symptoms were observed on Panniyur 6 as chlorotic spots after 55 days of grafting and a VI of 42 was recorded. Symptoms were recorded on Thevam after 58 days of grafting and the VI recorded was 32 (Plate 22). Shakti and Girimunda developed symptoms after 60 and 75 days of grafting with a vulnerability index of 28 recorded for both (Plate 23) On the basis of VI observed, Karimunda was categorized as a susceptible variety, while Panniyur 6, Shakti, Thevam, and Girimunda plants were categorized as moderately susceptible. In rest of the varieties no symptoms were observed till the 75^{th} day. Symptoms were not expressed in Panniyur varieties except Panniyur 6 and IISR Sreekara, IISR Subhakara and Panchami (Table 9).

4.4 SEROLOGICAL DIAGNOSIS AND MOLECULAR CHARACTERIZATION OF VIRUSES

Symptomatological studies cannot be the sole criterion to determine whether a plant is infected or not. Symptomatological studies must go in conjunction with serological and molecular detection procedures. Hence samples collected from surveyed plots were subjected to various serological and nucleic acid based detection methods.

a. Healthy varieties of black pepper taken for screening

b. Diseased scion collected from field

c. Wedge grafting

d. Grafted plants kept under shade nets for development of symptoms

Plate 20 (a-d). Screening of varieties for reaction to viruses

Plate 21. Symptom observed on grafted Karimunda plant 40 DAG

Plate 22. Symptom observed on grafted Thevam plant 58 DAG

Plate 23. Symptom observed on grafted Girimunda plant 75 DAG

Table 9. Response of varieties of black pepper to graft transmission of viruses

Sl. No	Variety	Days taken for symptom expression	Nature of symptom	VI	Inference
1	Karimunda	40	Chlorotic spots	48	S
2	Panniyur 6	55	Chlorotic spots	42	MS
3	Thevam	58	Chlorotic spots	32	MS
4	Shakti	60	Chlorotic spots	28	MS
5	Girimunda	75	Chlorotic spots	28	MS

S- Susceptible; S- Moderately susceptible

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

Serology based techniques like Enzyme Linked Immunosorbent Assay (ELISA) and Dot Immuno binding Assay (DIBA) were carried out. Double Antibody Sandwich ELISA (DAS-ELISA) was done using polyclonal antibodies of *Cucumber Mosaic Virus* (CMV) and two monoclonal antibodies of *Banana Streak Virus* (BSV). Since antibodies of *Piper yellow mottle virus* was not commercially available, antibodies of a serologically related virus was used for the detection. Both PYMoV and BSV belong to genus badnavirus and are serologically related also. From both Idukki and Wayanad, 20 samples with three replications were collected and analyzed. Primary antibody of CMV was used at a dilution of 1:1000 and that of BSV were used at 1: 200. Secondary antibodies were used at a dilution of 1: 10,000.

DAS-ELISA was found to be sensitive in detecting the presence of CMV and PYMoV from the diseased samples (Plate. 24). In Erattiyar panchayat none of the samples tested positive for CMV and one sample tested positive for BSV2 (Table 10). Out of the five samples tested in Kattappana panchayat three samples tested positive to CMV while one sample tested positive to both BSV1 and BSV 2. The samples tested positive for CMV had 2.2, 2.2 and 2 fold increases in OD value compared to healthy (0.018). The samples tested positive for BSV 1 and 2 recorded an absorbance value of 0.08 and 0.34 compared to healthy (0.04 for BSV 1and 0.091 BSV 2) which marks a 2 fold and 3.75 fold increase (Table 11). None of the samples tested positive for CMV in Pampadumpara panchayat while two samples tested positive for BSV1 (Table 12). The highest OD value recorded in Pampadumpara panchayat was 1.103 against OD value of 0.381 for healthy sample which was 2.8 times higher. The positive samples recorded an OD value of 0.803 and 1.1.03 which had a 2.1 and 2.8 fold increase when compared to healthy sample 0.381 (Table 12). No CMV infection was detected in samples collected from Karunapuram while two samples tested positive for BSV 1 (Table 13).

In Ambalavayal panchayat, none of the samples tested positive for CMV while three samples tested positive to BSV 2 and one sample tested positive for BSV 1 (Table 14). The absorbance value recorded for BSV 2 was 0.248 against an OD value of 0.06 for healthy sample which is 4.13 times more (Table 14). The sample tested positive to BSV 1 recorded an absorbance value of 0.785 against 0.336 recorded for healthy sample which is 2.3 times more. In Meenangadi panchayat, presence of CMV was detected in 3 samples, two samples tested positive to BSV1 and two samples tested positive to BSV 2. The samples tested positive for CMV recorded OD values of 0.042, 0.045 and 0.102 against a healthy sample with absorbance of 0.021 which shows a 2, 2.1 and 4.8 fold increase. One sample tested positive for both BSV 1 and BSV 2 which recorded an absorbance of 0.056 and 0.811 against an OD value of 0.026 and 0.38 for healthy sample (Table 15). From Meenangadi panchayat mixed infection of both viruses were detected in one sample.

The results of Poothadi panchayat detected the presence of CMV in two samples out of the five tested (Table 16). The highest OD value recorded was 0.09 against OD value of 0.02 in healthy samples which showed a 4.5 fold increase. Similarly in one sample presence of BSV1 and 2 were detected (Table 16). Mixed infections of both viruses were detected in the sample of Panamaram panchayat (Table 17). The highest OD value noticed was 0.08 in CMV infected sample against an OD value of 0.018 which shows a four times increase. Absorbance value of 0.231, 0.361 and 0.134 was recorded in samples tested for BSV 1 against 0.047 which shows 4.9, 7.6 and 2.85 fold increases respectively. Similarly for BSV 2 positive samples an OD value of 0.216 and 0.548 were recorded against 0.098 which shows a 2.20 and 5.6 fold increase. Out of the five samples tested, presence of CMV was detected in two samples and presence of BSV was detected in three samples.

Plate 24. Colour development in ELISA plate by interaction of virus infected samples with their respective antibodies

Table 10. Reaction of samples collected from Erattiyar panchayat to antibodies of CMV and BSV in DAS-ELISA

Samples	Mean* CMV	Fold increase in OD value	Mean* BSV 1	Mean* BSV 2	Fold increase in OD value	Inference
Healthy	0.710	-	0.001	0.070	-	-
Sample 1	1.200	1.70	0.002	0.096	1.40	-
Sample 2	1.239	1.77	0.006	0.093	1.32	-
Sample 3	1.164	1.64	0.007	0.139	1.98	BSV 2 +ve
Sample 4	1.007	1.42	0.003	0.029	0.40	-
Sample 5	1.221	1.72	0.005	0.027	0.40	-

+ = Positive reaction

* OD value calculated by taking mean of 3replication

Table 11. Reaction of samples collected from Kattappana panchayat to antibodies of CMV and BSV in DAS-ELISA

Samples	Mean* CMV	Fold increase in OD value	Mean* BSV 1	Fold increase in OD value	Mean* BSV 2	Fold increase in OD value	Inference
Healthy	0.018	-	0.040	-	0.091	-	-
Sample 1	0.040	2.20	0.014	0.35	0.069	0.75	CMV +ve
Sample 2	0.027	1.50	0.070	1.75	0.080	0.87	-ve
Sample 3	0.040	2.20	0.032	0.80	0.083	0.90	CMV +ve
Sample 4	0.019	1.05	0.080	2.00	0.340	3.73	BSV1, BSV2 +ve
Sample 5	0.036	2.00	0.050	1.25	0.026	0.20	CMV +ve

+ = Positive reaction

* OD value calculated by taking mean of 3 replications

Table 12. Reaction of samples collected from Pampadumpara panchayat to antibodies of CMV and BSV in DAS-ELISA

Samples	Mean* CMV	Mean* BSV 1	Fold increase in OD value	Mean* BSV 2	Fold increase in OD value	Inference
Healthy	0.035	0.381	-	0.097	-	-
Sample 1	0.050	0.803	2.1	0.087	0.80	BSV1+ve
Sample 2	0.020	0.698	1.8	0.094	0.96	-ve
Sample 3	0.020	0.712	1.8	0.104	1.07	-ve
Sample 4	0.024	1.103	2.8	0.073	0.75	BSV1 +ve
Sample 5	0.028	0.491	1.2	0.059	0.60	-ve

+ = Positive reaction

* OD value calculated by taking mean of 3 replication

Table 13. Reaction of samples collected from Karunapuram panchayat to antibodies of CMV and BSV in DAS-ELISA

Samples	Mean* CMV	Fold increase in OD value	Mean* BSV 1	Fold increase in OD value	Mean* BSV 2	Fold increase in OD value	Inference
Healthy	0.065	-	0.225	-	014	-	-
Sample 1	0.032	0.40	0.562	2.50	0.25	1.70	BSV1+ve
Sample 2	0.099	1.50	0.234	1.04	0.19	1.30	-
Sample 3	0.041	0.63	0.630	2.80	0.23	1.60	BSV1+ve
Sample 4	0.061	0.93	0.321	1.42	0.20	1.40	-
Sample 5	0.046	0.70	0.386	1.71	0.16	1.10	-

+ = Positive reaction

* OD value calculated by taking mean of 3 replications

Table 14. Reaction of samples collected from Ambalavayal panchayat to antibodies of CMV and BSV in DAS-ELISA

Samples	Mean* CMV	Fold increase in OD value	Mean* BSV 1	Fold increase in OD value	Mean* BSV 2	Fold increase in OD value	Inference
Healthy	0.052	-	0.336	-	0.060	-	-
Sample 1	0.054	1.03	0.373	1.10	0.137	2.20	BSV 2 +ve
Sample 2	0.046	0.80	0.377	1.10	0.140	2.30	BSV 2 +ve
Sample 3	0.057	1.09	0.385	1.10	0.061	1.01	-ve
Sample 4	0.042	0.80	0.785	2.30	0.098	1.60	BSV1 +ve
Sample 5	0.057	1.09	0.535	1.60	0.248	4.13	BSV2 +ve

+ = Positive reaction

* OD value calculated by taking mean of 3 replication

Table 15. Reaction of samples collected from Meenangadi panchayat to antibodies of CMV and BSV in DAS-ELISA

Samples	Mean* CMV	Fold increase in OD value	Mean* BSV 1	Fold increase in OD value	Mean* BSV 2	Fold increase in OD value	Inference
Healthy	0.021		0.026	-	0.38	-	-
-Sample 1	0.042	2.00	0.056	2.10	0.811	2.10	CMV,BSV 1&2 + ve
Sample 2	0.045	2.10	0.051	1.90	0.710	1.80	CMV,BSV1 +ve
Sample 3	0.102	4.80	0.017	0.60	0.720	1.80	CMV +ve
Sample 4	0.019	0.90	0.023	0.88	1.116	2.90	BSV2 +ve
Sample 5	0.020	0.90	0.009	0.30	0.43	1.10	-ve

+ = Positive reaction

* OD value calculated by taking mean of 3 replications

Table 16. Reaction of samples collected from Poothadi panchayat to antibodies of CMV and BSV in DAS-ELISA

Samples	Mean* CMV	Fold increase in OD value	Mean* BSV 1	Fold increase in OD value	Mean* BSV 2	Fold increase in OD value	Inference
Healthy	0.02	-	0.036	-	0.216	-	-
Sample 1	0.09	4.50	0.051	1.40	0.015	0.06	-ve
Sample 2	0.024	1.20	0.054	1.50	0.050	0.20	-ve
Sample 3	0.036	1.80	0.079	2.10	0.614	2.80	BSV1&2 +ve
Sample 4	0.07	3.50	0.055	1.50	0.037	0.10	-ve
Sample 5	0.015	0.75	0.012	0.30	0.037	0.10	-ve

+ = Positive reaction

* OD value calculated by taking mean of 3 replication

Table 17. Reaction of samples collected from Panamaram panchayat to antibodies of CMV and BSV in DAS-ELISA

Samples	Mean* CMV	Fold increase in OD value	Mean* BSV 1	Fold increase in OD value	Mean* BSV 2	Fold increase in OD value	Inference
Healthy	0.018		0.047	-	0.098	-	-
Sample 1	0.032	1.70	0.231	4.90	0.216	2.20	BSV 1&2 +ve
Sample 2	0.012	0.60	0.075	1.60	0.053	0.50	-ve
Sample 3	0.013	0.70	0.083	1.80	0.028	0.20	-ve
Sample 4	0.04	2.20	0.361	7.60	0.548	5.60	BSV 1&2 +ve
Sample 5	0.08	4.40	0.134	2.85	0.138	1.40	BSV 1+ve

+ = Positive reaction

* OD value calculated by taking mean of 3 replication

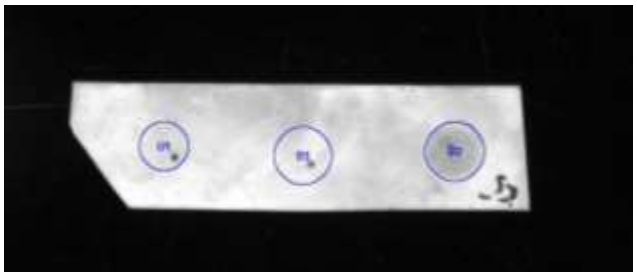
Infection of PYMoV was found to be predominant than CMV. Out of 40 samples tested ten samples were infected with CMV, 17 samples were infected with PYMoV and mixed infections were found in four samples.

4.4.2 Dot Immuno binding Assay

DIBA is another serology based detection method for the presence of viruses. DIBA was done to detect the presence of CMV and PYMoV using polyclonal antibodies of CMV and SCBV. SCBV antiserum is serologically related to PYMoV. CMV antiserum was used at a dilution of 1: 1000 and SCBV at a dilution of 1: 200. Secondary antibody was used at dilution of 1: 10,000. Development of a dark purple colour after the addition of substrate indicated the presence of virus (Plate 25). These samples were analyzed using gel documentation system (BIORAD Molecular Imager GEL DOCTM XR+). It was found that infected sample recorded higher mean value of intensity compared to healthy one. The mean value recorded using SCBV in Kattappana panchayat was 2023.2 for a diseased sample while for healthy it was 1556 (Table 18 and 19). Similar results were obtained for both the serological detection methods (Table 20). When the results of serological tests were compared with symptomatology it was found that mottling along with curling is associated with PYMoV infection. Reduction in leaf size and distortion of leaf were found associated with CMV infection (Plate 26).

a. CMV infected sample

b. PYMoV infected sample



U1- Healthy; U2- Diseased; B - Background

Plate 25. DIBA conducted for detection of CMV and PYMoV infected samples

Table 18. Reaction of stunted disease infected black pepper plants to respective polyclonal antibodies of CMV and SCBV

Sample	Label	Type	Min. Value (Int)	Max. Value (Int)	Mean ± Std Dev.
Erattiyar	SCBV	H	956	2777	1263.4 ± 188.5
		D	1165	2282	1571.6 ± 189.6
		B	1002	1891	1196.6 ± 86.3
Kattapana	CMV	H	1162	2498	1482.0 ± 178.0
		D	1058	3362	1551.1 ± 326.7
		B	1052	1850	1240.0 ± 110.3
	SCBV	H	1095	2243	1556.0 ± 154.6
		D	1437	2645	2023.2 ± 212.4
		B	1013	1926	1199.4 ± 84.2
Pampadumpara	SCBV	H	1059	2858	1378.7 ± 172.1
		D	1094	2306	1575.9 ± 20.4
		B	1002	1988	1220.0 ± 68.4
Karunapuram	SCBV	H	1296	2722	1529.9 ± 193.1
		D	1273	3204	1559.8 ± 200.3
		B	1098	2895	1302.0 ± 244.2
Ambalavayal	SCBV	H	1055	2417	1445.6 ± 191.6
		D	1245	2665	1552.9 ± 232.2
		B	1131	1910	1304.6 ± 89.6

Table 19. Reaction of stunted disease infected black pepper plants to respective polyclonal antibodies of CMV and SCBV

Sample	Label	Type	Min. Value (Int)	Max. Value (Int)	Mean ± Std. dev.
Meenangadi	CMV	H	1187	2956	1443.2±142.0
		D	1245	3426	1623.9±216.0
		B	969	3026	1188.0±144.8
	SCBV	H	1140	3275	1527.7±227.2
		D	229	3323	1916.9±231.2
		B	1067	3024	1401.9±198.9
Panamaram	CMV	H	1232	2740	1609.3±122.3
		D	1257	2991	2001.4±202.2
		B	973	2403	1190.7±103.1
	SCBV	H	1024	2886	1493.0±185.3
		D	1070	3144	1775.5±261.5
		B	1004	2809	1199.5±145.6
Poothadi	CMV	H	1135	3126	1391.0±181.1
		D	1180	3446	1895.9±318.1
		B	1173	3096	1379.8±191.3
	SCBV	H	973	3123	1246.8±219.0
		D	1162	2934	1903.2±293.1
		B	1181	1896	1413.6±83.1

Table 20. Consolidated results of ELISA and DIBA conducted for the samples collected from Idukki and Wayanad district

Sl. No.	Panchayat	Positive samples out of 5 collected samples			
		ELISA		DIBA	
		CMV	BSV	CMV	SCBV
1	Erattiyar	0	1	0	5
2	Pampadumpara	0	2	0	2
3	Kattappana	3	1	3	1
4	Karunapuram	0	2	0	2
5	Ambalavayal	0	4	0	4
6	Meenangadi	3	3	3	3
7	Poothadi	2	1	2	1
8	Panamaram	2	3	2	3

a. Mottling with curling associated with PYMoV infection

b. Reduction in leaf size associated with CMV infection

Plate 26 (a-b). Symptoms due to single infection of either CMV or PYMoV

4.4.3 Molecular characterization

Serology based detection methods of badnaviruses are less sensitive as they are highly heterogeneous at serological and genomic levels. Hence a nucleic acid based detection method becomes necessary which are more sensitive and reliable.

DNA was isolated from samples collected from surveyed locations showing the symptoms of PYMoV. DNA was isolated using CTAB (Cetyl tri methyl ammonium bromide) method. After washing with 70 per cent alcohol pelleted DNA was obtained. DNA was dissolved in 50µl of Tris EDTA buffer and stored.

4.4.4 Quantity and quality of DNA

The spectrophotometric analysis was done to check the quality and quantity of DNA obtained. The quality of DNA ranged from 1.11 to 1.41and the quantity ranged from 200 – 1000 ng μl^{-1}. Samples were collected from Idukki and Wayanad districts of Kerala. From Erattiyar quality of isolated DNA was 1.31, 1.30 and 1.12 and had a quantity of 415.4, 468.4 and 381.3 ng μl^{-1} respectively. Samples from Kattappana had a quality of 1.33, 1.18 and 1.24 and quantity of 146.5, 281.3 and 236.5 ng μl^{-1} respectively. Sample from Pampadumpara had a quality of 1.41 with a quantity of 518.2 ng μl^{-1}. DNA samples from Karunapuram recorded quality around 1.2-1.3 similar to that of Ambalavayal samples. Sample from Meenangadi had a quality around 1.43 with a quantity around 1110.3 ng μl^{-1}. Samples from Poothadi and Panamaram had quality of around 1.1- 1.3 with a quantity ranging from 250-500ng μl^{-1} (Table 21 and 22).

4.4.5 Sequencing and blast analysis

DNA samples with good quality and quantity were obtained. DNA with a quality of 1.3 to 1.4 and quantity not less than 250 ng μl^{-1} were taken for PCR. PCR amplification of ORF III was done using specific primers and agarose gel electrophoresis was performed. Amplicons of size 400 bp was obtained when compared with a 1 kb DNA ladder (Plate 27). In Idukki district, amplicon of size 473bp was

obtained from Erattiyar panchayat and 373bp from Kattappana panchayat. Similarly 378bp sequence was obtained from Pampadumpara region and amplicon size of 378bp size was obtained from Karunapuram region. In Wayanad district, amplicons of 380bp was obtained from Ambalavayal, and a 424bp sequence from Meenangadi region. In Poothadi and Panamaram sequences were 383bp and 511 bp respectively. The virus isolates were subjected to BLAST (Basic Local Alignment Search Tool) analysis after sequencing. Regions of local similarity between protein and nucleotide sequences were analyzed using BLAST. On blast analysis, it was found that PYMoV isolate from Erattiyar was more similar to *Piper yellow mottle isolate* PN P1 from Kodagu, Karnataka (96.24 percent), Kattappana isolate was showing 97.93 per cent similarity to *Piper yellow mottle isolate* PN P1, Pampadumpara isolate had 98.84 per cent similarity to *Piper yellow mottle isolate* PN P1and Karunapuram isolate was 93.20 per cent similar to *Piper yellow mottle virus isolate* PN6 isolated from Hassan, Karnataka (Table 23).

Samples from Wayanad upon blast analysis found that, Ambalavayal isolate had 97.46 per cent similarity *to Piper yellow mottle virus* from India polyprotein gene, partial cds; Meenangadi isolate had 93.97 per cent similarity with *Piper yellow mottle virus from India* polyprotein gene, partial cds; Poothadi isolate was showing 97.16 per cent similarity to *Piper yellow mottle virus from India* polyprotein gene, partial cds and 97.67 per cent similarity to *Piper yellow mottle virus from India* polyprotein gene, partial cds; and Panamaram isolate was found to have 98.59 per cent similarity with *Piper yellow mottle virus from India* polyprotein gene, partial cds (Table 24).

BLAST analysis results of Erattiyar panchayat showed 96.24 per cent similarity to *Piper yellow mottle isolate* PN P1 from Kodagu, Karnataka, *and Piper yellow mottle virus* strain ISH-1 from India. Similarity around 95.97 per cent was observed with *Piper yellow mottle virus* isolate PN6 polyprotein gene, partial cds (Table 25). Isolates of Kattappana showed 97.93 per cent similarity to both *Piper yellow mottle isolate* PN P1 and *Piper yellow mottle virus* strain ISH-1. Similarity around 97. 64 and 97.34 per cent

was observed with *Piper yellow mottle virus* isolate PN6 and *Piper yellow mottle virus* isolate PO1 polyprotein gene, partial cds from Kozhikode, Kerala (Table 26).

Isolates of Pampadumpara showed 98.84 per cent similarity to *Piper yellow mottle virus* isolate PN P1 Complete genome, *Piper yellow mottle virus* strain ISH-1 and *Piper yellow mottle virus* from India (Table 27).

Karunapuram isolate showed 93.20 per cent similarity to both *Piper yellow mottle virus* isolate PN6 and *Piper yellow mottle virus* isolate from India whereas 92.90 per cent similarity was obtained with both *Piper yellow mottle virus* isolate PN9 from Kannur, Kerala and *Piper yellow mottle virus* isolate PN-P1. Similarity to other badna virus were also found 93.18 per cent similarity to *Citrus yellow mosaic virus* isolate SO JNTU, complete genome, 81.25 per cent similarity to *Banana streak* (Table 28).

Ambalavayal isolate had 97.46 per cent similarity with *Piper yellow mottle virus* from India polyprotein gene, DQ836227.1 and 97.61 per cent similarity with *Piper yellow mottle virus* from India polyprotein gene, DQ836229.1. Similarity to *Musa ABB Group endogenous virus* badnavirus clone KT6 ORFIII polyprotein-like gene (79.47 per cent) was recorded (Table 29).

Isolate from Meenangadi showed 93.97 per cent similarity to *Piper yellow mottle virus* from India polyprotein gene, partial cds, DQ836229.1 and 93.50 per cent similarity to *Piper yellow mottle virus* from India polyprotein gene, partial cds, DQ836227.1 (Table 30).

Poothadi isolate recorded more similarity to *Piper yellow mottle virus* from India (97.67 per cent) (Table 31). Panamaram isolate had 98.59 per cent similarity to *Piper yellow mottle virus* from India polyprotein gene, partial cds, and 78.31 per cent similarity to *Musa ABB Group endogenous virus* badnavirus clone KT6 ORFIII (Table 32).

Table 21. Quality and quantity of DNA isolated from infected leaf samples in Idukki district recorded using spectrophotometer

Sl. No.	Isolate name	A260	A280	260/280value	Quantity of DNA (ng μl^{-1})
1	E1	0.083	0.063	1.31	415.4
2	E2	0.094	0.072	1.30	468.4
3	E3	0.076	0.067	1.12	381.3
4	P1	0.103	0.073	1.41	518.2
5	P2	0.090	0.072	1.25	450.8
6	P3	0.089	0.074	1.19	448.2
7	K1	0.029	0.021	1.33	146.5
8	K2	0.056	0.047	1.18	281.3
9	K3	0.047	0.038	1.24	236.5
10	KA1	0.089	0.065	1.37	446.4
11	KA2	0.085	0.063	1.33	427.6
12	KA3	0.066	0.051	1.29	331.9

E- Erattiyar; P- Pampadumpara; K- Kattappana; KA- Karunapuram

Table 22. Quality and quantity of DNA isolated from infected leaf samples in Idukki district recorded using spectrophotometer

Sl. No.	Isolate name	A260	A280	260/280value	Quantity of DNA (ngμl^{-1})
1	A1	0.137	0.099	1.38	689.5
2	A2	0.107	0.095	1.12	537.4
3	A3	0.057	0.045	1.25	289.4
4	M1	0.147	0.109	1.34	736.1
5	M2	0.222	0.155	1.43	1110.3
6	M3	0.150	0.135	1.11	754.1
7	P1	0.107	0.086	1.23	536.4
8	P2	0.043	0.042	1.01	216.7
9	P3	0.106	0.080	1.31	532.3
10	PA1	0.098	0.071	1.38	494.8
11	PA2	0.058	0.043	1.34	291.3
12	PA3	0.088	0.074	1.18	444.3

A- Ambalavayal; M- Meenangadi; P- Poothadi; PA- Panamaram

Plate 27. Electrophoresis gel image of amplified DNA of PYMoV infected samples from Idukki and Wayanad, lane M- 1000 bp DNA ladder; lane 1-8: isolates from Erattiyar, Kattappana, Pampadumpara, Karunapuram, Ambalavayal, Meenangadi, Poothadi and Panamaram panchayats.

Table 23.NCBI BLAST analysis for sequences similarity of PYMoV isolate from survey locations in Idukki with other PYMoV isolates reported in black pepper

Sl. No.	Isolate	Similarity to other badnavirus sequences	Percentage similarity	Accession number
1	Erattiyar	*Piper yellow mottle virus* isolate PN P1 Complete genome	96.24%	KJ873041.1
		Piper yellow mottle virus strain ISH-1,complete genome	96.24%	KC808712.1
2	Karunapuram	Pipe yellow mottle virus isolate PN6	93.20%	KJ195468.1
		Piper yellow mottle virus isolate from India polyprotein gene, partial cds	93.20%	DQ836227.1
3	Kattappana	*Piper yellow mottle virus* isolate PN P1 Complete genome	97.93%	KJ873041.1
		Piper yellow mottle virus strain ISH-1,complete genome	97.93%	KC808712.1
4	Pampadumpara	*Piper yellow mottle virus* isolate PN P1 Complete genome	98.84%	KJ873041.1
		Piper yellow mottle virus strain ISH-1,complete genome	98.84%	KC808712.1

Table 24. NCBI BLAST analysis for sequences similarity of PYMoV isolate from survey locations in Wayanad with PYMoV isolates reported in black pepper

Sl. No.	Isolate	Similarity to other badnavirus sequences	Percentage similarity	Accession number
1	Ambalavayal	*Piper yellow mottle virus* from India polyprotein gene, partial cds	97.46%	DQ836227.1
		Piper yellow mottle virus from India polyprotein gene, partial cds	96.91%	DQ836229.1
2	Meenangadi	*Piper yellow mottle virus* from India polyprotein gene, partial cds	93.97%	DQ836229.1
		Piper yellow mottle virus from India polyprotein gene, partial cds	93.50%	DQ836227.1
3	Poothadi	*Piper yellow mottle virus* from India polyprotein gene, partial cds	97.16%	DQ836227.1
		Piper yellow mottle virus from India polyprotein gene, partial cds	97.67%	DQ836229.1
4	Panamaram	*Piper yellow mottle virus* from India polyprotein gene, partial cds	98.59%	DQ836227.1
		Piper yellow mottle virus isolate PN P1 Complete genome	97.77%	KJ873041.1

Table 25. NCBI BLAST analysis for sequences similarity of PYMoV isolate from Erattiyar, Idukki with other badnavirus isolates

Description	Percentage similarity	Accession number
Piper yellow mottle virus isolate PN P1 Complete genome	96.24%	KJ873041.1
Piper yellow mottle virus strain ISH-1,complete genome	96.24%	KC808712.1
Piper yellow mottle virus from India polyprotein gene, partial cds	96.24%	DQ836229.1
Piper yellow mottle virus isolate PN6 polyprotein gene, partial cds	95.97%	KJ195468.1
Piper yellow mottle virus from India polyprotein gene, partial cds	95.97%	DQ836231.1

Table 26.NCBI BLAST analysis for sequences similarity of PYMoV isolate from Kattappana, Idukki with other badnavirus isolates

Description	Percentage similarity	Accession number
Piper yellow mottle virus isolate PN P1 Complete genome	97.93%	KJ873041.1
Piper yellow mottle virus strain ISH-1,complete genome	97.93%	KC808712.1
Piper yellow mottle virus from India polyprotein gene, partial cds	97.93%	DQ836229.1
Piper yellow mottle virus isolate PN6 polyprotein gene, partial cds	97.63%	KJ195468.1
Piper yellow mottle virus isolate PO1 polyprotein gene, partial cds	97.34%	KJ195487.1

Table 27. NCBI BLAST analysis for sequences similarity of PYMoV isolate from Pampadumpara, Idukki with other badnavirus isolates

Description	Percentage similarity	Accession number
Piper yellow mottle virus isolate PN P1 Complete genome	98.84%	KJ873041.1
Piper yellow mottle virus strain ISH-1,complete genome	98.84%	KC808712.1
Piper yellow mottle virus from India polyprotein gene, partial cds	98.84%	DQ836229.1
Piper yellow mottle virus isolate PN6 polyprotein gene, partial cds	98.55%	KJ195468.1
Piper yellow mottle virus from India polyprotein gene, partial cds	98.55%	DQ836231.1

Table 28. NCBI BLAST analysis for sequences similarity of PYMoV isolate from Karunapuram, Idukki with other badnavirus isolates

Description	Percentage similarity	Accession number
Piper yellow mottle virus isolate PN6	93.20%	KJ195468.1
Piper yellow mottle virus isolate from India polyprotein gene, partial cds	93.20%	DQ836227.1
Piper yellow mottle virus isolate PN9 polyprotein gene, partial cds	92.90%	KJ195471.1
Piper yellow mottle virus isolate PN-P1, complete genome	92.90%	KJ873041.1
Banana streak VN virus isolate E1063-29 reverse transcriptase-ribonuclease H gene, partial cds	81.25%	KF318357.1
Citrus yellow mosaic virus isolate SO JNTU, complete genome	93.18%	JN006805.1

Table 29. NCBI BLAST analysis for sequences similarity of PYMoV isolate from Ambalavayal, Wayanad with other badnavirus isolates

Description	Percentage similarity	Accession number
Piper yellow mottle virus from India polyprotein gene, partial cds	97.46%	DQ836227.1
Piper yellow mottle virus from India polyprotein gene, partial cds	96.91%	DQ836229.1
Piper yellow mottle virus isolate PNP1, complete genome	96.90%	KJ873041.1
Musa ABB Group endogenous virus badnavirus clone KT6 ORFIII polyprotein-like gene, partial sequence	79.47%	AY452270.1

Table 30. NCBI BLAST analysis for sequences similarity of PYMoV isolate from Meenangadi, Wayanad with other badnavirus isolates

Description	Percentage similarity	Accession number
Piper yellow mottle virus from India polyprotein gene, partial cds	93.97%	DQ836229.1
Piper yellow mottle virus from India polyprotein gene, partial cds	93.50%	DQ836227.1
Musa ABB Group endogenous virus badnavirus clone KT6 ORFIII polyprotein-like gene, partial sequence	79.66%	AY452270.1
Sweet potato badnavirus B isolate Hunan-184, complete genome	95.24%	MK052981.1

Table 31. NCBI BLAST analysis for sequences similarity of PYMoV isolate from Poothadi, Wayanad with other badnavirus isolates

Description	Percentage similarity	Accession number
Piper yellow mottle virus from India polyprotein gene, partial cds	97.16%	DQ836227.1
Piper yellow mottle virus from India polyprotein gene, partial cds	97.67%	DQ836229.1
Piper yellow mottle virus isolate PN-P1, complete genome	97.38%	KJ873041.1
Piper yellow mottle virus strain ISH-1, complete genome	97.38%	KC808712.1
Musa ABB Group endogenous virus badnavirus clone KT6 ORFIII polyprotein like gene, partial sequence	77.78%	AY452270.1

Table 32. NCBI BLAST analysis for sequences similarity of PYMoV isolate from Panamaram, Wayanad with other badnavirus isolates

Description	Percentage similarity	Accession number
Piper yellow mottle virus from India polyprotein gene, partial cds	98.59%	DQ836227.1
Piper yellow mottle virus isolate PN P1 Complete genome	97.77%	KJ873041.1
Piper yellow mottle virus strain ISH-1,complete genome	97.77%	KC808712.1
Piper yellow mottle virus from India polyprotein gene, partial cds	98.03%	DQ836229.1
Musa ABB Group endogenous virus badnavirus clone KT6 ORFIII polyprotein like gene, partial sequence	78.31%	AY452270.1

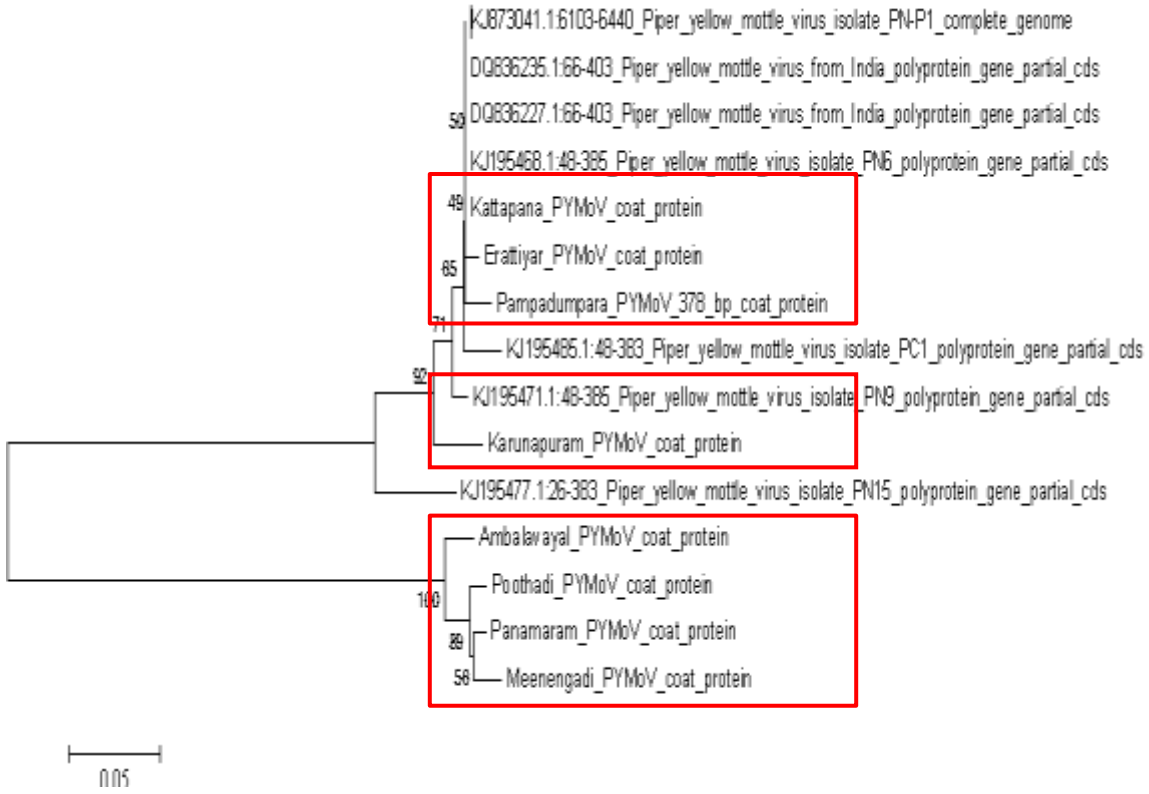


Plate 28. Phylogeny tree showing the virus isolates in three different clades

4.4.6 Construction of Phylogeny tree

Phylogenetic analysis is helpful in understanding the process of evolution. Phylogeny tree were constructed with the MEGA 6 software. Phylogenetic studies revealed that isolates from Idukki and Wayanad were grouped in separate clades. Isolates from Erattiyar, Kattappana and Pampadumpara were clustered together indicating they are closely related. These isolates were showing similarity to *Piper yellow mottle virus* isolate PC1 polyprotein gene, partial cds obtained from Kannur, Kerala. Karunapuram isolate was found distantly related with the other three Idukki isolates. Isolates from Ambalavayal, Meenangadi, Poothadi and Panamaram were grouped in another clade (Plate 28).

The study revealed that viral diseases of black pepper are predominant in the major pepper growing areas of black pepper. Serological techniques like DAS-ELISA and DIBA could be used for detection of these viruses along with molecular detection using coat protein specific primers. Varietal screening conducted revealed that Karimunda was the most susceptible variety. The isolates of PYMoV from different locations of Idukki and Wayanad showed similarity in CP gene to other reported PYMoV sequences. Sensitive detection methods and proper management measures need to be adopted to manage the disease under field conditions.

Discussion

5. DISCUSSION

Black pepper, known as King of Spices is a major spice crop cultivated in Kerala. Incidence of viral diseases has become a serious threat limiting the cultivation and production of black pepper. Black pepper being a vegetatively propagated crop, chances of spreading the viral disease is high. Hence, the following study was undertaken with the objectives to study the occurrence of the viruses infecting black pepper, its sero-molecular detection and to study the varietal response to infection by the viruses. In this chapter we are discussing about the results of the study undertaken.

5.1 SURVEY

A survey was conducted during May 2019- February 2020 in two districts of Kerala viz. Idukki and Wayanad to study the incidence and severity of viral diseases. These two districts were selected for surveying as black pepper is a prominent spice crop cultivated in these districts.

5.1.1 Disease incidence, Vulnerability index and Presence of vectors

A total of eight panchayats and 40 farmer fields were surveyed for studying the incidence and severity of viral diseases in black pepper. The incidence of viral disease was recorded in all the panchayats surveyed (Figure 1). It was found that the highest disease incidence was recorded at Poothadi panchayat (45.50 per cent) in Wayanad district and lowest incidence at Karunapuram panchayat (21.62) of Idukki district. The severity of disease was expressed in terms of vulnerability index (Figure 2). High vulnerability index were recorded from Poothadi (58) and Panamaram (61) panchayats of Wayanad districts (Table 5 and 6).

Bhat *et al.* (2005b) conducted studies on the stunted disease incidence in Karnataka and Kerala. Highest disease incidence was found in Wayanad (45.4 per cent) and Idukki (29.4 per cent) districts of Kerala. Studies conducted at Kodagu and Hassan

district of Karnataka recorded a disease incidence of 14.9 per cent and 5.2 per cent disease incidence in Hassan district (Bhat *et al*., 2005). The results of survey were in conformity with these observations.

Parthasarathy *et al*. (2005) observed that as the altitude varied between 750–800 m and between 900-1500 m in Wayanad and Idukki respectively, the disease incidence varied between 45 per cent and 90 per cent. But in the present survey, more disease incidence and disease severity were observed in Wayanad than Idukki which is in contrary to the above results (Figure 1 and 2). Disease incidence at Pampadumpara panchayat located at 1200 m elevation recorded a disease incidence of 29.72 per cent. However, Poothadi panchayat of Wayanad situated at 765m elevation recorded an incidence of 45.50 per cent. Disease incidence was higher in poorly managed fields than well maintained fields.

Association of a *Cucumber mosaic virus* was reported with black pepper in many pepper growing areas. Bhat *et al*. (2008) reported the disease occurrence as either due to pure infection by CMV or PYMoV or due to mixed infection by both the viruses. The intensity of disease was found more in case of mixed infections. Mixed infections of the viruses have been reported in a variety of vegetable crops and it is seen that combined infections leads to increased severity. Often other infection had the symptoms of only one virus and masks the symptoms of the other. When mixed infections lead to an increased severity, it is referred to as synergistic disease. Few of the synergistic diseases reported are corn lethal necrosis in corn (Niblett and Clafin, 1978), and cowpea stunt (Pio- Reibero *et al*., 1978).

Mixed infection in maize is caused by *Maize chlorotic mottle virus* and either *Maize dwarf mosaic virus* or *Wheat streak mosaic virus* or *Maize dwarf mosaic virus* where the symptoms are varying degrees of mosaic due to infection by different viruses. Another report is the infection Zucchini squash plants infected with CMV and *Zucchini yellow mosaic virus.* Mixed infection of *Sweet potato feathery mottle virus* and *Sweet*

potato chlorotic stunt virus also developed severe symptoms. Synergistic interaction of CMV and *Pepper mottle virus* (Potyvirus) was established in *Capsicum annum* (Murphy and Bowen, 2005). In the present study, mixed infection was observed in Panamaram panchayat of Wayanad district. The high disease severity observed in Panamaram can be substantiated due to the detection of both viruses on serological diagnosis (Table 17). Stunted growth of vines were found associated with CMV while mottling along with curling was found on infection with PYMoV.

Selvarajan *et al*. (2009) studied the mitigation of banana bract mosaic disease and compensation of yield loss through higher dose of fertilizer application. Umadevi *et al*. (2016) reported severe symptom expression in plants growing under poor soil nutrient status and temperature stress. In the present survey, increased disease incidence was found in poorly managed fields than in well maintained fields which is in concurrence with the above mentioned results.

Ayisha (2010) conducted the survey in two districts of Kerala viz., Kollam and Trivandrum and found that the viral diseases of black pepper were predominant in the southern parts of Kerala. The present study was focused on the severity of the viral diseases in the major pepper growing areas and it could be inferred that the disease is wide spread in major pepper growing tracts of Idukki and Wayanad which warrants immediate strategies to be adopted for the management of stunted disease.

In the present study, presence of mealy bugs was found associated with viral infected plants in the surveyed locations of Idukki and Wayanad (Plate 5 and 6). Transmission studies conducted by De Silva (1996) confirmed mealy bug, *Planococcus citri* (Risso) as vectors of PYMoV. Horizontal spread of badnavirus is by means of mealy bugs. However due to the slow movement of mealy bugs, insect transmission is not considered to be an efficient method of spread under field conditions (Daniells *et al*, 2001). Ayisha (2010) has reported the successful transmission of the virus through *Ferrisia virgata*.

5.1.2 Maintenance of inoculum

Virus infected plants were kept under insect proof shade nets for further studies at College of Agriculture, Vellayani (Plate 7). A reduction in symptom expression was observed in some plants after a rain and increased expression was observed during summer months. Symptomless nature was reported for some other members of badnaviruses like *Sugarcane mosaic virus* by Lockhart and Autrey (1988), *Banana streak virus* by Lockhart (1995) and for *Citrus yellow mosaic virus* by Baranwal *et al.* (2003).

Masking of symptoms in black pepper was observed by De Silva *et al*. (2002) during winter and monsoon season. Bhat *et al*. (2005a) have opined that expression of symptoms were at the best during March to May which coincides with summer season. Bhat *et al*. (2012) reported the symptomless nature of PYMoV in black pepper varieties like Panchami, Panniyur 1 and Panniyur 5. It was found that these varieties even though remain as symptomless, act as a source of secondary spread in the field by means of vector transmission and grafting.

5.2 SYMPTOMATOLOGY

Symptomatological studies were undertaken for proper identification of disease in the field. Symptoms observed in different survey locations were chlorotic spots, vein banding, distortion and reduction in leaf size, general chlorosis, puckering of leaves and vein clearing (Plate 8). Symptoms were observed on both older and younger leaves. In Karunapuram panchayat of Idukki, only mild symptoms like chlorotic specks were observed. The disease incidence observed in Karunapuram panchayat was also less (Plate 12). Symptoms were severe in poorly managed fields without proper fertilization

and weeding. Adoption of good cultivation practices and proper fertilization might help in the management of disease.

Lockhart (1997) observed mild to severe chlorotic mottling, vein-clearing, interveinal chlorosis, reduction in leaf size and leaf puckering and deformation. Stunted disease caused by PYMoV was characterized by chlorotic mottling, vein clearing, distortions of leaves, reduced plant vigour, chlorosis and poor fruit set (Durate *et al*., 2001). Infection by PYMoV is characterized by vein clearing, scattered chlorotic flecks followed by chlorotic mottling along veins leading to interveinal chlorosis and characteristic curling of the leaves. It was found that vein banding, vein thickening and green island-like symptoms are also seen. The infected vines had reduced vigour and yield (Bhat *et al.*, 2005b). Ayisha (2010) observed small chlorotic spots on young leaves which later becomes small, sickle shaped, leathery and had stunted growth. Vein clearing, mottling, curling and twisting of leaves were also observed. Similar symptoms were recorded in all the survey locations in which mottling symptom with curling was found to be predominant (Plate 8 to 16).

Sarma *et al*. (2001) observed symptoms like interveinal yellow flecking, yellow mottling, dark-green vein banding, narrow distorted leathery leaves and overall reduction in growth associated with CMV infection in black pepper. Stunting, and reduction and distortion of leaf size were found associated with the infected samples collected from Kattappana, Poothadi and Panamaram panchayats where the presence of CMV was identified based on serology (Plate 26). Stunting was less frequently observed, while reduction and distortion of leaf size was found in samples collected from the above listed panchayats. In some samples, an admixture of all the described symptoms were observed indicating mixed infection of viruses. Hence symptomatology alone cannot be used for the successful detection of viruses emphasizing the need for serological and molecular detection techniques.

Natural incidence and severity of the disease was found higher in variety Karimunda. Karimunda and Kottanadan belonged to highly susceptible category and Neelamundi was found to be susceptible. Shakthi, Panniyur 1 and Thekkan pepper belonged to moderately susceptible category and no visible symptoms were observed on Thekkan pepper plants grafted with *Piper coloubrinum* (Figure 3 and 4*).*

Varietal screening was conducted by Ayisha (2010) in which out of 13 varieties observed 61.4 per cent incidence was observed in Karimunda plants. Disease incidence recorded in Panchami and Pournami was cent per cent while no symptom was observed on Cherumaniyan variety cultivated in Thenmala, Kollam district. Disease incidence recorded in Neelamundi was 40 per cent and that of Panniyur and Shakti were 43 and 40 per cent respectively. In the present study increased incidence was found associated with these varieties (Neelamundi and Shakti 50 per cent each; Panniyur- 54 per cent) (Table 5 and 6).

Thekkan pepper grafted on *Piper coloubrinum* did not express any symptoms of the disease. *P. coloubrinum* might be a source of resistance or a symptomless carrier of virus. Ayisha (2010) conducted a varietal screening in which diseased scions were graft inoculated to *P. coloubrinum* and was found to be symptomless. Researchers have attributed the resistance as due to increase in defense related enzymes. On analysis of PPO (Poly phenol oxidase) activity, it was found that infected leaves of Karimunda scion grafted on *P. coloubrinum* had higher PPO activity compared to naturally infected Karimunda plants and concluded as the root stock effect of *P. coloubrinum.* Bhat *et al.* (2012) identified the symptomless nature of *P. coloubrinum* and confirmed that the virus transmission could occur from these symptomless sources to healthy plants through vectors and grafting. Hence, they act as a source of secondary spread in the field conditions.

Symptoms due to infection of badnaviruses were found varying with climate, varieties and environmental factors. Banana streak disease caused by *Banana streak*

virus is characterized by the chlorotic or necrotic streaks, pseudostem splitting. However, it was found that the symptoms remission occurs during certain time period followed by reappearance of symptoms. Severe symptoms were found on banana plants grown at 22°C but became mild and weaker as and when transferred to 28-30°C (Dahal *et al*, 1998). Similar observations are found with the case of PYMoV. Umadevi*et al*. (2016) studied the effect of temperature and changes in host factors associated with symptom expression. Influence of temperature on symptom expression was analyzed based on the changes in the expression of host proteins, virus titre and other physiological parameters. Plants were subjected to temperature stress and PCR was done before and after experiment to detect the presence of virus. Plants tested negative for PCR before stress showed the presence of virus in low copy number when subjected to temperature stress. Through this investigation, it was concluded that masking of symptoms may be due to the variations in temperature.

5.3 SCREENING FOR RESISTANCE

Rooted cuttings of Panniyur varieties (1 to 8), IISR varieties like Sreekara, Subhakara, Thevam, Shakthi, Girimunda, Panchami and local cultivar Karimunda with three replications were maintained. Wedge shaped cut was made on the infected scion using a sharp knife and a slit cut was made on the root stock. Grafted plants were kept under insect proof conditions for observation (Plate 20). The varietal screening was done on the basis of days taken for symptom expression, nature of symptom development and vulnerability index. The healthy plants of 15 varieties were grafted with the infected scions of Karimunda and observed under insect proof conditions. On the basis of VI observed Karimunda was categorized as a susceptible variety, while Panniyur 6, Shakti, Thevam, Girimunda plants were categorized as moderately susceptible (Table 9). In rest of the varieties no symptoms were observed till the 75^{th} day. Panniyur 6 which is a clonal selection from Karimunda expressed symptoms after 55 days of grafting, Thevam which is a clonal selection from Thevanmundi expressed

symptoms after 58 days of grafting (Arya *et al.*, 2003). IISR Shakthi, an open pollinated progeny form Perambramundi developed symptoms after 60 days of grafting. IISR Girimunda which is a hybrid of Narayakodi and Neelamundi developed symptoms after 75 days of grafting. Neelamundi was categorized as a susceptible variety in the present study when surveyed for natural disease incidence.

Bhat *et al*. (2003) conducted graft transmission studies where the infected scions were cleft grafted to healthy black pepper seedlings raised from seeds. Symptoms appeared in 2-3 months of grafting. Bhat *et al*. (2005) screened a total of 2186 black pepper nursery plants belonging to eleven varieties by DAS-ELISA for the presence of viruses. Infection caused by either CMV or PYMoV was detected in 714 plants. Three replications were maintained for each variety and young leaf collected from each plant during the first week of every month was analyzed for the presence of the virus using DAS-ELISA. Among them, highest number of infected plants was found with variety IISR-Sreekara followed by IISR Subhakara, while Panniyur-5 had the least number of infected plants. IISR Sreekara and Subhakara were clonal selections from Karimunda which might be responsible for the susceptibility associated with it. Of the 714 plants tested positive, 390 plants exhibited no visible external symptoms indicating the necessity for sensitive diagnosis in identifying virus-free planting material. In the present study also, symptom expression was not observed in Panniyur 6, Sreekara, Subhakara and Panchami.

Bhat *et al.* (2012) conducted graft transmission studies where scions top cleft grafted on to rootstock plants. It was found that within 30 days of grafting, symptom expression was recorded in 50 per cent of Karimunda plants. Symptom development was recorded in 70 per cent Karimunda plants on 60 days after grafting and after 90 days of inoculation symptom was found on 94 per cent plants.

Works of many researchers have pointed out the susceptibility of the variety Karimunda to viral infection. Our study also has shown the susceptibility of the var.

Karimunda to virus infection as the plants grafted with infected scion could take up infection within a short time of 40 days. The expression of symptoms in Panniyur 6 could also be attributed to its lineage to Karimunda as it is a clonal selection of Karimunda. Bhat *et al*. (2012) identified the symptomless nature of PYMoV associated with varieties of Panniyur 1, Panniyur 5 and Panchami also confirmed the presence of virus using specific primers.

Ayisha (2010) conducted varietal screening where twelve varieties were tested for their resistance to viruses and the assessment was done based on vulnerability index on 0-5 scale. Wedge grafting was done using the infected scions of Karimunda. It was found that Panniyur 2, 3 and 4 were moderately resistant and moderate susceptibility was recorded for Panniyur 5, 6, 7, Malabar excel and Shakthi. Kuthiravallypadappan and Kottanadan were found susceptible while Karimunda was highly susceptible. Karimunda recorded the highest vulnerability index 52 followed by Kuthiravallypadan and Kottanadan with a recorded VI of 47 and 42 respectively.

5.4 SEROLOGICAL DIAGNOSIS AND MOLECULAR CHARACTERIZATION OF VIRUSES

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

ELISA could be employed when there are information regarding the viruses involved in causing the infection. The antigen-antibody interaction is detected using a substrate and intensity of colour development could be transformed as numerical values in ELISA reader. Bhadramurthy (2012) analyzed leaf samples of black pepper and found infections with CMV and PYMoV. In the present study samples collected from different survey locations were subjected to DAS-ELISA using polyclonal antiserum of CMV-Banana (1: 1000) and monoclonal antiserum of BSV 1 and BSV 2 (1: 200). Bhat *et al*. (2003) used the antisera of different badna virus and found that none of the samples

reacted positively to RTBV (*Rice Tungro Bacilliform Virus*) and CoYMV (*Commelina Yellow Mottle Virus*) antiserum while all samples had a positive reaction towards BSV and SCBV antisera. CoYMV is the type species of badna virus and RTBV belongs to genus tungro virus, family Caulimoviridae. RTBV and CoYMV were found serologically not related to PYMoV while BSV and SCBV were found closely related on serology basis. Since the availability of PYMoV antisera is meagre; it was imperative to try for other related antiserum viz., BSV and SCBV.

In Erattiyar panchayat none of the samples tested positive for CMV but all the samples were positive to BSV1 and one sample tested positive for BSV 2. The mean absorbance value (OD value) 0.007 was observed for infected plants compared to mean OD value 0.001 (7 times more) recorded for healthy plants in Erattiyar panchayat (Table 10). Out of the 5 samples tested in Kattappana panchayat, three samples tested positive to CMV while one sample tested positive to both BSV1 and BSV 2 (Table 11). None of the samples tested positive for CMV in Pampadumpara panchayat while two samples tested positive for BSV1. The highest OD value recorded in Pampadumpara panchayat was 1.103 against OD value of 0.381 for healthy sample which was 2.8 times higher (Table 12). No CMV infection was detected in samples collected from Karunapuram (Table 13).

In Ambalavayal panchayat, none of the samples tested positive for CMV while three samples tested positive to BSV 2 and one sample tested positive for BSV. The absorbance value recorded for BSV 2 was 0.248 against an OD value of 0.06 for healthy sample which is 4.13 times more (Table 14). Regarding Meenangadi panchayat presence of CMV was detected in 3 samples, two samples tested positive to BSV1 and two samples tested positive to BSV 2. From Meenangadi panchayat mixed infection of both viruses were detected in one sample (Table 15). The results of Poothadi panchayat detected the presence of CMV in two samples out of the five tested (Table 16). Mixed infection of both viruses was detected in the sample of Panamaram panchayat (Table

17). Out of the five samples tested, presence of CMV was detected in two samples and presence of BSV was detected in three samples. In the present study positive reaction was obtained with CMV (Banana) and BSV which is in conformity with previous studies undertaken. Infection of PYMoV was found to be predominant than CMV. Out of 40 samples tested 10 samples were infected with CMV, 21 samples were infected with PYMoV and mixed infection was found in four samples (Table 20).

Sarma (2001) in his study has highlighted the use of polyclonal antibodies for the quick detection of viruses. The infected samples reacted positively to indirect antigen coating-ELISA and electro blot immunoassay tests with polyclonal antisera of CMV-Banana (India), CMV-Brinjal (India), CMV-Chilli (India), CMV-Tomato (India), CMV-L (USA) and CMV-A (China). Bhat *et al*. (2004) standardized DAS-ELISA for detection of CMV in infected black pepper samples. Bhadramurthy *et al* (2005) reported a standard DAS ELISA technique for the detection of a badnavirus in black pepper. It was possible to identify infected samples from the healthy samples with an increase in the O.D. values. However, some of the samples exhibiting symptoms of the disease gave an O.D. close to that of the healthy samples due to low titre value. In the present study, some samples expressing symptoms were found to have low absorbance value which might have been due to this low titre value. The genomic and serological heterogeneity of badnaviruses can be a reason for lesser sensitivity of serological methods. The varying OD values found in the positively tested samples indicate the varied concentration of virus at different locations.

Comparison of symptoms with the serological results obtained in ELISA showed that mottling with curling is associated with infection of PYMoV whereas, reduction and distortion in leaf size was observed with CMV infection (Plate 26). Ayisha (2010) observed that leaves showing general chlorosis reacted positively to both CMV and PYMoV while sickle shaped leaves were found to be infected with

CMV. Leaf samples with severe chlorosis and mottling found reacted to SCBV, which was serologically related to PYMoV.

5.4.2 Dot Immunobinding Assay

DIBA is yet another serology based detection method for viral infections in plants. DIBA was done to detect the presence of CMV and PYMoV using polyclonal antibodies of CMV and SCBV. CMV antiserum was used at a dilution of 1:1000 and SCBV at a dilution of 1:200. Development of a dark purple colour after the addition of substrate indicated the presence of virus (Plate 25). It was found that infected sample recorded higher mean value of intensity compared to healthy one on analysis with gel doc system (Table 18 and 19) . This confirms the serological relationship of PYMoV with SCBV. Ayisha (2010) used polyclonal antibodies of CMV and SCBV and found it successful for the detection of viruses.

Banttari and Goodwin (1985) detected PVS, PVX and PVY by using dot ELISA on nitrocellulose membranes at respective dilutions of 1:16000, 1:128000 and 1:16000. Powell (1987) reported a dot immuno binding assay for detection of *Tobacco mosaic virus* (TMV), *Tobacco ring spot virus* (TbRSV) and *Tomato ring spot virus* (TmRSV). This method was found more sensitive than DAS- ELISA and also helps in rapid detection of the infected viruses.

5.4.3 Molecular characterization

DNA was isolated from leaf samples collected from different survey locations showing the symptoms of PYMoV. DNA isolation was done using CTAB method with slight modification where incubation was done overnight after addition of Isopropanol. Bhat *et al*. (2008) isolated total DNA slightly modifying the protocol described by De Silva *et al*. (2002) where phenol: chloroform: isoamyl alcohol (25:24:1) was used.

Amplification of ORF III was done using specific primers. Hany *et al*. (2013) studied the complete genome sequence of PYMoV with 7.2 kb size. The genome consists of four open reading frames (ORFs) with ORF 1, 2 and 4 having molecular mass of 15.7, 17.1 and 17.9 kDa. ORF3 of PYMoV is a polyprotein of 218.6 kDa which encodes a viral movement protein (MP), trimeric dUTPase, zinc finger, retropepsin, RT-LTR, and RNAse H. Coat protein region is not well demarcated in badna viruses. Hence ORF III was used for amplification.

Delanoy *et al*. (2003) reported the use of real time PCR for detection of BSOLV (*Banana streak obino l'Ewai virus*) and BSMYV *(Banana streak mysore virus*). Selvarajan *et al.* (2016) developed a detection method raising polyclonal antibodies using recombinant VAP (Virus Associated Protein) encoded by ORFIII which would help to detect different strains of BSV. Wambulwa *et al.* (2012) made detection of BSV more reliable using multiplex immuno capture PCR (M-IC-PCR) and rolling circle amplification (RCA)

5.4.4 Quantity and quality of DNA

The spectrophotometric analysis was done to check the quality and quantity of DNA obtained. The quality of DNA ranged from 1.11 to 1.40 and the quantity ranged from 200 – 1000 ng μl^{-1} (Table 21 and 22). Pelleted DNA was obtained on overnight incubation on addition of Isopropanol. Bhat *et al*. (2009) standardized the template volume of DNA needed to get good amplification. For this PCR was carried out using different template volumes (0.25µl, 0.5 µl. 1.0µl and 5.0 µl). It was found that 0.5 µl and 1.0 µl were found optimum. In the present study template volume of 1.0 µl was found to give good DNA amplification.

5.4.5 Sequencing and blast analysis

PCR amplification of ORF III was done using specific primers and agarose gel electrophoresis was conducted. Amplicons of size 400 bp was obtained when compared with a 1 kb DNA ladder (Plate 27). Bhat *et al.* (2008) conducted PCR detection based on the ORF III sequence of the PYMoV infecting black pepper in India (GenBank accession No. DQ836227) and for a positive reaction, bands of 400 bp were obtained.

On blast analysis, it was found that PYMoV isolate from Erattiyar was more similar to *Piper yellow mottle isolate* PN P1 (96.24 percent) (Table 25), Kattappana isolate was showing 97.93 per cent similarity to *Piper yellow mottle isolate* PN P1 (Table 26), Pampadumpara isolate had 98.84 per cent similarity to *Piper yellow mottle isolate PN P1* (Table 27*)* and Karunapuram isolate was 93.20 per cent similar to *Piper yellow mottle virus isolate* PN6 (Table 28).

Samples from Wayanad upon blast analysis, it was found that Ambalavayal isolate had 97.46 per cent similarity *to Piper yellow mottle virus* from India polyprotein gene, partial cds (Table 29), Meenangadi isolate had 93.97 per cent similarity with *Piper yellow mottle virus from India* polyprotein gene, partial cds (Table 30), Poothadi isolate was showing 97.16 per cent similarity to *Piper yellow mottle virus from India* polyprotein gene, partial cds and 97.67 per cent similarity to *Piper yellow mottle virus from India* polyprotein gene, partial cds (Table 31), and Panamaram isolate was found to have 98.59 per cent similarity with *Piper yellow mottle virus from India* polyprotein gene, partial cds (Table 32).

Using badnavirus specific primers, Lockhart and Olszewski (1994) amplified regions of PYMoV. Lockhart (1997) found ORF III to be closely related to other mealybug-transmitted badnaviruses like badnaviruses BSV, CoYMV and SCBV. They were found distantly related to caulimoviruses *viz.*, *Cauliflower mosaic virus* (CaMV), *Enation itched ring* (CERV), *Figwort mosaic* (FMV), *Soyabean chlorotic mottle*

(SoyCMV). Similar results were obtained in the present study where similarity to SCBV and BSV was recorded on blast analysis. Badnaviruses are known to infect many crops like banana and cocoa; which are found in a mixed cropping system along with black pepper. Hence the similarity of PYMoV to other badnavirus needs to be studied further to understand whether these viruses can act as an infection source to black pepper.

De Silva *et al*. (2002) used primers SCBV R1 and Badna-T to amplify the expected amplicon of 700 bp. Hany *et al*. (2013) reported that ORF2 of PYMoV showed around 44 per cent identity to CYMV (NP-569152) and 39 per cent identity to DBV (AB147985.1); while ORF3 was having 50 per cent similarity to ORF3 of DBV (YP-001036293) and 55 per cent identity to CYMV (NP-569153). In the present study also, higher similarity of these viruses were found.

Bhat and Siju (2007) developed a single-tube multiplex RT–PCR for the simultaneous detection of CMV and PYMoV in black pepper. PCR primers designed for amplifying 650 bp from the coat protein gene of CMV and 450 bp from the open reading frame I of PYMoV were used. The method is rapid, reliable and aids in the rapid screening of a large number of plants infected by both the viruses. PCR amplification of viral DNA was obtained with primer for BSV and a 500bp amplicon size was obtained (Ayisha, 2010).

5.4.6 Construction of phylogeny tree

Phylogenetic analysis was done to study the similarities and evolutionary relationships between the isolates. Phylogenetic studies revealed that isolates from Idukki and Wayanad grouped in separate clades. Isolates from Erattiyar, Kattappana and Pampadumpara were clustered together indicating they are closely related. Karunapuram isolate was found distantly related with the other three Idukki isolates. Isolates from Ambalavayal, Meenangadi, Poothadi and Panamaram were found as another clade (Plate 28). Phylogenetic analysis showed that PYMoV is closely related

to *Cacao swollen shoot virus* (CSSV), *Citrus yellow mosaic virus* (CYMoV) and *Dioscorea bacilliform virus* (DBV) (Hany *et al*., 2014).

Through this study, it was concluded that viral infections are prevalent in cultivated areas of Idukki and Wayanad districts of Kerala. Infection of CMV and PYMoV occur either in pure form or as mixed infections. Serological techniques like DAS-ELISA and DIBA along with molecular detection using coat protein specific primers could be used for routine detection of these viruses. Varietal screening undertaken revealed that Karimunda was a susceptible variety. The isolates of PYMoV from different locations of Idukki and Wayanad showed similarity to other reported PYMoV sequences. Development of sensitive and reliable techniques needs to be done for proper management of disease. Sustainable management of CMV and PYMoV using endophytes and nutrients need to be explored. *P. coloubrinum* as source of resistance needs to be investigated.

Summary

6. SUMMARY

The present research work entitled 'Varietal screening of black pepper to *Cucumber mosaic virus* and *Piper yellow mottle virus* and their sero-molecular detection' was carried out at the Department of Plant Pathology, College of Agriculture, Vellayani during the period 2018- 2020 with the objectives to study the occurrence and sero-molecular detection of *Cucumber mosaic virus* (CMV) and *Piper yellow mottle virus* (PYMoV) infecting black pepper and to identify the varietal response to infection by the viruses.

Survey was conducted in the two major pepper growing districts of Kerala, Idukki and Wayanad. A total of eight panchayats were surveyed for assessing the incidence and severity of the viral diseases in black pepper. From Idukki district two blocks *viz.*, Kattappana and Nedumkandam blocks were selected. The four panchayats selected from two blocks were Erattiyar and Kattappana, and Pampadumpara and Karunapuram, repectively. From Wayanad district, Panamaram and Bathery blocks were selected and the panchayats selected were Poothadi, Panamaram, Ambalavayal and Meenangadi. Survey conducted in these locations revealed that viral diseases of black pepper are wide spread in these regions. Disease incidence varied from 21- 37 per cent in Idukki district while it varied between 24- 45 per cent in Wayanad district. Highest disease incidence was recorded in Erattiyar panchayat (37.40 %) and lowest in Karunapuram panchayat (21.62 %); whereas in Wayanad district highest incidence was recorded at Poothadi (45.50 %) and lowest in Meenangadi panchayat (24.50 %). Highest vulnerability index was recorded in Erattiyar panchayat (45) of Idukki district while lowest was recorded at Pampadumpara (32). In Wayanad, highest VI was recorded in Panamaram (61) and lowest in Ambalavayal (37).

Diseased samples were collected from the surveyed locations to study the symptomatology of virus infection. Various symptoms observed in the field were chlorotic spots, vein banding, distortion and reduction in leaf size, general chlorosis,

puckering of leaves and vein clearing. Symptoms initially appear as small chlorotic spots and gradually leading to interveinal chlorosis, vein clearing, severe mottling and vein banding. Reduction in leaf size, distortion in shape of leaf and stunting of growth with slight mottling were also observed in the virus infected plants. In the field condition (natural incidence), the varieties Karimunda and Kottanadan were found to be highly susceptible to the viruses; the variety Neelamundi was found to be susceptible; and the varieties Shakti, Panniyur 1 and Thekkan pepper were categorized as moderately susceptible to the viruses. No visible symptoms were observed on Thekkan pepper plants grafted with *Piper coloubrinum*.

A trial was laid out to study the varietal response to infection of the viruses. Healthy plants of 15 varieties were grafted with infected scions of Karimunda and observed for expression of symptoms under insect proof conditions. The parameters for screening were days taken for symptom expression after grafting, nature of symptom development and vulnerability index. Studies on varietal reaction indicated that the variety Karimunda could be categorized under the susceptible category, while Panniyur 6, Shakti, Thevam, and Girimunda plants were categorized as moderately susceptible. Rest of the varieties taken for study did not produce any visible symptoms till 75^{th} day after grafting.

Serological technique *viz*., double antibody sandwich-ELISA (DAS-ELISA) was done using polyclonal antibodies of *Cucumber Mosaic Virus* (CMV) and two monoclonal antibodies of *Banana Streak Virus* (BSV). From both Idukki and Wayanad, 20 samples with three replications were collected and analyzed. Primary antibody of CMV was used at a dilution of 1:1000 and that of BSV were used at 1:200. Secondary antibodies were used at a dilution of 1:10,000. DAS-ELISA was found to be sensitive in detecting the presence of CMV and PYMoV from the diseased samples. Infection of PYMoV was found to be predominant than CMV. Out of 40 samples tested 10 samples were infected with CMV, 21 samples were infected with PYMoV and mixed infection was found in 4 samples. DIBA was done to detect the

presence of CMV and PYMoV using polyclonal antibodies of CMV and *Sugarcane bacilliform virus* (SCBV). CMV antiserum was used at a dilution of 1:1000 and SCBV at a dilution of 1:200. Secondary antibody was used at dilution of 1:10,000. Samples tested positive for the virus were indicated by the development of purple colour in respective area of sample placement. The results were analyzed using gel documentation system. It was found that mottling along with curling is associated with PYMoV infection; reduction and distortions in leaf size were found associated with CMV infection which was confirmed using serological tests.

Molecular detection was carried out using specific primers for ORF III region of badnaviruses. DNA was isolated using CTAB method and amplification was done as per specifications. The amplified PCR products were subjected to sequencing and blast analysis. All the isolated sequences were found to be related to PYMoV. Phylogenetic studies revealed that isolates from Idukki and Wayanad grouped in separate clades. Isolates from Erattiyar, Kattappana and Pampadumpara were clustered together indicating that they are closely related while isolates from Ambalavayal, Meenangadi, Poothadi and Panamaram formed a separate clade.

References

7. References

Alif, T., Hartono, S., and Sulandari, S. 2018. Characterization of viral causes of striped disease in pepper plants (*Piper nigrum L*.). *J. Perl. Tan. Indo*. 22: 115-123.

Alles, W.S. 1984. Little leaf disease of pepper. Information note no. 2. In: *Land Potential for Coffee, Oil Crops and Spice Crops in Ethiopia, A Consultancy Report.* UNDP/FAO project, Sri Lanka/77/009. Rome: FAO.

Arya, K., Rajagopalan, A., Satheesan, K.N., Nair, P.K.U., Mammotty, K.P., Zacharia, G., and Vanaja, T. 2003. Panniyur-6 and Panniyur-7 high yielding black pepper elections for Kerala. *Indian J. Genetics and Plant Breeding*, 63(4): 363-364.

Ayisha, R. 2010.Characterisation and management of viral diseases of Black pepper (*Piper nigrum* L.) PhD (Ag) Thesis, Kerala Agricultural University, Thrissur, 115p.

Banttari, E.E. and Goodwin, P.H. 1985. Detection of potato viruses S, X, and Y by enzyme-linked immunosorbent assay on nitrocellulose membranes (Dot-ELISA). *Plant Dis.*, 69(3): 202-205.

Baranwal, V.K., Majumder, S., Ahlawat Y.S., and Singh, R. P. 2003. Sodium sulphite yields improved DNA of higher stability for PCR detection of *Citrus yellow mosaic virus* from citrus leaves. *J. Virol. Meth*. 112:153–156.

Bhadramurthy, V., Bhat, A.I., Thankamani, J.G.C., and Mathew, P.A. 2008. Variation in the concentration and indexing black pepper plants for PYMoV and CMV through DAS-ELISA. *J. of Spices and Aromat. Crops* 17(2):197-201.

Bhadramurthy, V., Ratheesh, S.T., Bhat, A.I., Madhubala, R., Hareesh, P.S., and Pant, R. P. 2005. Development of ELISA- based technique for the detection of a putative badnavirus infecting black pepper (*Piper nigrum* L.). *Indian Phytopathol*. 58: 314-318.

Bharat, H. 1952. A study on decline of the pepper plantations in Indo-China. *Arch. Rech. Agron. Cambodage Laos et Vietnam* 13: 75-92.

Bhat A.I., Hareesh, P.S., and Thomas, T. 2003. Viral diseases of some important spice crops In: Characterization, Diagnosis, and Management of viral diseases. Vol 2 *Horticultural crops*, Studium press, LLC USA: pp 223- 248.

Bhat, A.I, Devasahayam, S., Hareesh, P.S., Preethi, N., and Thomas, T. 2005c. *Planococcus citri* (Risso) -an additional mealybug vector of *Badnavirus* infecting black pepper (*Piper nigrum* L.) in India. *Entomon* 30: 85-90.

Bhat, A.I., Devasahayam, S., Sarma, Y. R., and Pant, R. P. 2005a. Association of a Badnavirus in black pepper (*Piper nigrum* L.) transmitted by mealy bug (*Ferrisia virgata*) in India. *Curr. Sci*. 84: 1547-1550.

Bhat, A.I. and Siju, A. 2014. Detection of viruses infecting black pepper by SYBR green based real time PCR assay. *J. of Plant Pathol. 6*(1): 105-109.

Bhat, A.I. and Siju, A. 2007. Development of a single-tube multiplex RT-PCR for the simultaneous detection of *Cucumber mosaic virus* and *Piper yellow mottle virus* associated with stunt disease of black pepper. *Curr. Sci*. 93(7): 973-976.

Bhat, A.I., Biju, C.N., Srinivasan, V., Ankegowda, S.J., and Krishnamurthy, K.S. 2017. Current status of viral diseases affecting black pepper and cardamom. *J. Spices Aromat. Crops* 27(1): 01-16.

Bhat, A.I., Devasahayam, S., Venugopal, M.N., and Suseela bhai, R. 2005b. Distribution and incidence of viral diseases of black pepper (*Piper nigrum* L.) in Kerala and Karnataka. *Indian J. Plant. Crops* 33: 16-21.

Bhat, A.I., Faisal, T.H., Madhubala, R., Hareesh, P.S., and Pant, R.P. 2004. Purification, production of antiserum and development of enzyme linked immunosorbent assay -based diagnosis for *Cucumber mosaic virus* infecting black pepper (*Piper nigrum* L.). *J. Spices Aromat. Crops* 13(1): 16-21.

Bhat, A.I., Sasi, S., Deeshma, K.P., and Saji, K.V. 2014. Sequence diversity among Badnavirus isolates infecting black pepper and related species in India. *Virus Dis*. 25: 402-407.

Bhat, A.I., Siju, A., and Devasahayam, S. 2012. Occurrence of symptomless source of *Piper yellow mottle virus* in black pepper (*Piper nigrum* L.) varieties and a wild *Piper* species. *Arch. Phytopathol. Plant Prot.* 45(9): 1000-1009.

Bhat, A.I., Siju, A., Jiby, M.V., Thankamani, C.K., and Mathew, P.A. 2009. Polymerase Chain Reaction (PCR) based indexing of Black pepper *(Piper nigrum L*.) plant against *Piper yellow mottle virus*. *J. Spices Aromat. Crops* 18(1): 28-32.

Bhat, A. I. and Siju, S. Identification and characterization of a badna virus infecting betel vine and Indian long pepper. 2008. *J. Plant Biochemistry*, 17(1), pp.73-76.

Borah, B.K., Johnson, A.M., Sai Gopal, D.V., and Dasgupta, I. 2009. Sequencing and computational analysis of complete genome sequences of *Citrus yellow mosaic badnavirus* from acid lime and pomelo. *Virus Genes*. 39:137–140.

Bos, L., 1982. Crop losses caused by viruses. *Crop Protection, 1*(3), pp. 263-282.

Caner, J. and Ikeda, H. 1972. A virus disease of black pepper in St. Paulo state. *Biologica* 38: 93-94.

Costa, A.S, Carneiro de Albuquerque, F., Ikeda, H., and Cardosa, M. 1969. Black Pepper Mosaic Caused by *Cucumber Mosaic Virus*, Series: Phytotechnics. Campinas, Brazil: Institute of Agricultural Research and Experimentation of the North diseases of black pepper in Sarawak. pp. 44–46.

Dahal, G., Hughes, J.D.A., Thottappilly, G., and Lockhart, B.E.L. 1998. Effect of temperature on symptom expression and reliability of *banana streak badnavirus* detection in naturally infected plantain and banana (*Musa* spp.). *Plant Dis*. 82: 16–21.

Daniells, J.W., Geering, A.D., Bryde, N.J., and Thomas, J.E. 2001. The effect of Banana streak virus on the growth and yield of dessert bananas in tropical Australia. *Ann. Appl. Biology 139*(1): 51-60.

Delanoy, M., Salmon, M., Kummert, J., Frison, E., and Lepoivre, P. 2003. Development of real-time PCR for the rapid detection of episomal Banana streak virus (BSV). Plant Dis. 87: 33–38.

De Silva, D. P. P., Jones, P., and Shaw, M. W. 2002. Identification and transmission of *Piper yellow mottle virus* and *Cucumber mosaic virus* infecting black pepper (*Piper nigrum* L.) in Sri Lanka. *Plant Pathol.* 51: 537 – 545.

De Silva, D.P.P., Jones, P., and Shaw, M.W. 2001. Isolation, purification and identification of *Cucumber mosaic virus* (CMV) in Sri Lankan black pepper (*Piper nigrum* L.). *Sri Lankan J. of Agric. Sci.* 38: 17 – 27.

De Silva, D.P.P. 1996. Studies of black pepper (*Piper nigrum* L.) virus disease in Sri Lanka. PhD Thesis. University of Reading, UK, 215 p.

Deeshma, K.P. and Bhat, A.I., 2014. Further evidence of true seed transmission of *Piper yellow mottle virus* in black pepper. (*Piper nigrum* L.) *J. Plant. Crops* pp.2898-293.

Doyle, J.J. and Doyle, J.L. 1990. Isolation of plant DNA from plant tissue. Focus 12(1) life technical Inc. pp. 13-15.

Duarte, M. L. R., Albuquerque, F. C., and Chu, E. Y. 2001. New diseases affecting black pepper crop in Brazil. *Int. Pepper Bull*. pp. 51–57.

Eng, L., Jones, P., Lockhart, B., and Martin, R.R. 1993. *Preliminary studies on the virus diseases of black pepper in Sarawak.* p. 149-161.

Firdausil, A.B., Rusmilah, S., and Sitepu, D. 1992. Stunted disease of black pepper. In: *Proceedings of International Workshop on Black Pepper Diseases,* pp. 220-225.

Hany, U., Adams, I.P., Glover, R., Bhat, A.I., and Boonham, N. 2014. The complete genome sequence of *Piper yellow mottle virus* (PYMoV). *Arch. of virol.* 159(2): 385-388.

Hareesh, P.S. and Bhat, A.I. 2008. Detection and partial nucleotide sequence analysis of *Piper yellow mottle virus* infecting black pepper in India. *Indian J. Virol.* 19(2): 160-167.

Herbers, K., Tacke, E., Hazirezaei, M., Krause, K.P., Melzer, M., Rohde, W., and Sonnewald, U. 1997. Expression of a luteoviral movement protein in transgenic plants leads to carbohydrate accumulation and reduced photosynthetic capacity in source leaves. *Plant J.* 12(5): 1045-1056.

Holliday, P. 1959. Suspected virus in black pepper. *Common Phytopathol. News* 5: 49-54.

IISR [Indian Institute of Spice Research]. 2005. *Annual report* 2004-2005. Indian Institute of Spice Research, Calicut: 96p

Johnson, A.M., Borah, B.K., Sai Gopal, D.V., and Dasgupta, I. 2012. Analysis of full-length sequences of two *Citrus yellow mosaic badnavirus* isolates infecting *Citrus jambhiri* (Rough Lemon) and *Citrus sinensis* L. Osbeck (Sweet Orange) from a nursery in India. *Virus Genes*. 45:600–605.

Kueh, T.K. and Sim, S.L. 1992. Occurrence and management of wrinkled-leaf disease of black pepper In: *Proceedings of the International Workshop on Black Pepper Diseases* pp. 227–233.

Kumar, S., Tamura, K., and Nei, M. 1994. MEGA: Molecular Evolutionary Genetics Analysis software for microcomputers. *Bioinforma*. 10(2): 189-191.

Lakani, R. 2006. Detection and identification of the causes of the Belang disease (Mottle) in pepper plants (*Piper nigrum* L.) in Indonesia.

Lockhart, B.E.L and Autrey, L.J.C. 1988. Occurrence in sugarcane of a bacilliform virus related serologically to *Banana streak virus*. *Plant Dis*. 72: 230–273.

Lockhart, B.E.L. 1995. Banana streak *Badnavirus* infection in Musa: epidemiology, diagnosis and control. Food Fertilizer Technol. *Center Tech Bull*. 143:11

Lockhart, B. E. L. and Olszewski, N. E. 1994. Badnavirus Group. In: *Encyclopedia of Virology* Vol. I, Academic Press, New York, pp. 139-143.

Lockhart, B. E. L. 1997. Identification of *Piper yellow mottle virus*, a mealy bug transmitted badnavirus infecting *Piper spp*. *Southeast Asia. European J. Plant Pathol*. 103: 303- 311.

Lockhart. B.E. 1993. University of Minnesota, USA. Personal communication.

Medberry, S.L., Lockhart, B.E., and Olszewski, N.E. 1990. Properties of *Commelina yellow mottle virus*'s complete DNA sequence, genomic discontinuities and transcript suggest that it is a pararetrovirus. *Nucleic Acids Res.* 18: 5505–5513.

Miftakhurohmah, Hidayat, S. H., Mutaqin, K. H., Soekarno, B. P. W., and Wahyuno, D. 2020. Incidence and severity of mottle disease in black pepper plants (*Piper nigrum* L.) in Sukamulya Research Station, West Java. IOP. 418(1).p.012054.

Murphy, J.F. and Bowen, K.L. 2006. Synergistic disease in pepper caused by the mixed infection of *Cucumber mosaic virus* and *Pepper mottle virus*. *Phytopathol*. 96(3): 240-247.

NHB (National Horticulture Board), 2016. URL. http://nhb.gov.in/annual report.aspx

Niblett, C. L. and Claflin, L. E. 1978. Corn lethal necrosis- a new virus disease of corn in Kansas. Plant Dis. Rep. 62:15-19.

Paily, P. V., Remadevi, L., Nair, V. G., Menon, M. R., and Nair, M. R. G. K. 1981. Malformation of leaves in black pepper. *J. Plantn. Crops* 9: 61-62.

Palukaitis, P., Roossinck, M., Dietzgen, R. G., and Francki, R.I.B. 1992. *Cucumber mosaic virus. Adv. Virus Res*. 41: 281-348.

Parthasarathy, U., Bhat, A.I., Devasahayam, S., Jayarajan, K., and Parthasarathy, V.A. 2005. Pepper pots throw light on stunted disease IISR Annual report, Indian institute of spice research, Calicut pp.220–226.

Pio-Ribeiro, G., Wyatt, S. D., and Kuhn, C. W. 1978. Cowpea stunt: A disease caused by a synergistic interaction of two viruses. *Phytopathology* 68:1260-1265.

Lalwani, S., Pooniya, S., Raina, A., Millo, T., and Dogra, T.D., 2014. Quality and quantity of extracted deoxyribonucleic Acid (DNA) from preserved soft tissues of putrefied unidentifiable human corpse. *J. Lab. Physicians*, 6(1): 31.

Powell, C.A., 1987. Detection of three plant viruses by dot-immunobinding assay. *Phytopathology*, 77(2): 306-309.

Prakasam, V., Subbaraja, K.T., and Bhakthavatsalu, C.M. 1990. Mosaic disease-a new record in black pepper in Lower Palneys. *Indian Cocoa, Arecanut and Spices J. 13*(3): 104.

Randombage, S. and Bandra, J. M. R. S. 1984. Little leaf disease of *Piper nigrum* in Srilanka. *Plant Pathol*. 33: 479-482.

Sarma, Y.R., Kiranmai G., Sreenivasulu P., Anandaraj M., Hema M., Venkatramana M., Murthy A.K. and Reddy, D.V.R., 2001. Partial characterization and identification of a virus associated with stunt disease of black pepper (*Piper nigrum*) in South India. *Curr Sci.* 80: 459-462.

Sarma, Y. R., Anandaraj, M., and Devasahayam, S. 1992. Diseases of unknown etiology of black pepper (*Piper nigrum* L.). In: *Proc. Internat. Workshop on Black pepper diseases* (eds. Wahid, P., Sitepu, D., Deciyanto, S. and Superman, U. (Eds). Inst. for Spice Med. Crops, Bogor, Indonesia. pp. 133-143.

Selvarajan, R., Balasubramanian, V., Jeyabaskaran, K.J., Pandey, S.D., and Mustaffa, M.M. 2009. Management of banana bract mosaic disease through higher dose of fertilizer in banana cv. Ney Poovan (AB). *Indian J. Hortic*. 66 (33): 301-305.

Selvarajan, R., Balasubramanian, V., and Gayathrie, T. 2016. Highly efficient immunodiagnosis of episomal Banana streak MY virus (BSMYV) using polyclonal antibodies raised against recombinant viral associated protein. *J. Phytpathol*. 164. (7): 497-508.

Siju, S., Madhubala, R., and Bhat, A.I. 2008. Sodium sulphite enhances RNA isolation and sensitivity of *Cucumber mosaic virus* detection by RT-PCR in black pepper. *J. Virol. Methods* 141(1): 107-110.

Sitepu, D. and Kasim, R. 1991. Black pepper diseases in Indonesia and their control strategy. In: *Black Pepper Diseases.* Sarma, Y. R. and Premkumar, T. (Eds). National Research Centre for Spices, Calicut, pp. 13–28.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30: 2725-2729.

Senthil Kumar, T.S. and Uma Swarupa, P. 2017. Cultivation Practices of Black Pepper in India – A Review. *International Journal of Innovative Research in Management Studies* 2(5):21-27.

Umadevi, P., Bhat, A. I., Krishnamurthy, K. S., and Anandaraj, M. 2016. Influence of temperature on symptom expression, detection of host factors in virus infected *Piper nigrum* L. *Indian J. Exp. Biol.* 54(5): 354-360.

Wahid, P., Sitepu, D., Deciyanto, S. and Ujang Superman, D. 1992. In: Proceedings of the International Workshop on Black Pepper Agency for Agricultural Research and Development Research Institute for Spices and Medical Crops, Bogor, Indonesia, .

Wambulwa, M.C., Wachira, F.N., Karanja, L.S., and Muturi, S.M. 2012.Rolling circle amplification is more sensitive than PCR and serology-based methods in detection of Banana streak virus in Musa germplasm. *Am. J. Plant Sci.* 3: 1581–1587.

Appendices

APPENDIX I

Buffers for DAS-ELISA

1. Phosphate buffer saline (PBS-pH- 7.4)

Sodium chloride -8.0g
Potassium dihydrogen phosphate -0.2g
Disodium hydrogen phosphate -1.1g
Potassium chloride -0.2g
Sodium azide -0.2g
Water -1 L

2. Wash buffer (PBS-T)

Add 250µL of Tween 20 to 500 ml of PBS

3. Coating buffer (pH 9.6)

Sodium carbonate -1.59 g
Sodium bicarbonate -2.93g
Sodium azide -0.2g
Water -1 L

4. Antibody diluents buffer

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

5. Enzyme conjugate diluents buffer

Same as PBS-TPO

6. Substrate solution (pH 9.8)

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

APPENDIX II
Buffers for DIBA

1) Stock solution buffer (Tris Buffer Saline, pH 7.5)

0.02 M Tris - 4.84 g
0.5 M NaCl -58.48 g
Adjust pH to 7.5 with 1N HCl and make up to 2 litres, this can be used as washing solution.

2) Antigen extracting buffer (TBS-SDM)

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

3) Blocking Solution

Spray dried milk 5 g
TBS buffer 100 ml
Add 5g of spray dried milk to 100ml of TBS

4) Antibody and enzyme- conjugate diluent/ buffer

Add antibody of required diluents to TBS-SDM

5) Substrate buffer (pH 9.5)

0.1 M Tris 12.11g
0.1 M NaCl 5.85 g
5 mM $MgCl_2$. $6H_2O$ 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 (DMF) -1 ml

Solution B

Bromo chloro indolyl phosphate (BCIP) - 50 mg
Dimethyl formamide (DMF) -1 ml

7) Fixing solution (pH 7.5)

10mM Tris 1.21 g
1mM 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 III

DNA isolation

1) CTAB DNA extraction buffer (pH 8.0)

100 mM Tris-Cl (pH 8.0)

25 mM EDTA (pH 8.0)

2 M NaCl

2% (w/v) CTAB

Add 2% (w/v) β –mercaptoethanol and 2% (w/v) Polyvinylpyrrolidone (PVP) at the time of grinding.

2) TE buffer (pH 8.0)

Tris- 10 mM

EDTA – 1 mM

3) Chemicals and solutions

CTAB buffer – 2 ml

Chloroform: Iso Amyl Alcohol (24:1) – 500 µl

Isopropanol- 500 µl

70% Alcohol-100 µl

TE buffer- 50 µl

Appendix IV

Buffers for PCR products and Gel electrophoresis

50X TAE buffer

Tris Base – 242 g

Glacial Acetic Acid – 57.1 ml

0.5 M EDTA (pH 8.0) – 100 ml

Make up the volume to 1L using distilled water

10 X TAE buffer

0.1 M Tris-Cl
0.01 M EDTA
Prepare 800 ml of distilled water in a suitable container

Add 15.759 g of Tris-Cl (desired pH) to the solution

Add 2.92 g of EDTA (pH 8.0) to the solution

Make up the volume to 1L using distilled water

6X Gel loading dye

30% (v/v) Glycerol

0.25% (w/v) Bromophenol blue

0.25% (W/V) Xylene cyanol FF

1.2% agarose is prepared for casting and examination of PCR products

Appendix V

PYMoV isolate from Erattiyar Panchayat

Erattiyar (473 bp)

TCTAGTGGTTGTACGAGCTAGTGTTCTAAGAATATGTACAACTGTTTCAAGG
TATGGAAGATTTTATAGCTGTCTACATTGATGATATTCTGGTGTTCTCAGAAA
ACATGAGGGACCATGCCCAACACTTAGTCGCAATGCTGGAAGTATGCAAGA
AGAAGGGGCTTATCTTAAGCCCAACAAAAATGAAAATTGGGCTTGGAACTA
TTGATTTCCTGGGAGCAACTATTGGAAACAGCAAGGTAAAGCTACAAGAGC
ACATCGTTAAGAAAATCCTGGACTTCAATACTGATGGTCTCGAAGACAAAA
AGAATCTCCGGAGTTGGCTGGTTATTCTCAACTATGCAAGGGCATACATACC
AAACCTAGGAAATCCTTGGCCCTCTCTTGTGATGTCAGGCCAAAAAAGCTC
CGAGTCGGTGCCCTTTTTCGAGTTTATAACGGACTAACCTTATTTTATCTTGT
TATTTCTA

Appendix VI

PYMoV isolate from Kattappana panchayat

Kattappana (373 bp)

AATTTTAATTGCTGGTGTCGTGCTGTGTCACGAAGAGGACACTGTTTCAAG
GATGGAAATTTTATAGCTGTCTACATTGATGATATTCTGGTGTTCTCAGAAAA
CATGAGGGACCATGCCCAACACTTAGTCGCAATGCTGGAAGTATGCAAGAA
GAATGGGCTTATCTTAAGCCCAACAAAAATGAAAATTGGGCTTGGAACTATT
GATTTTCTGGGAGCAACTATTGGAAACAGCAAG
GTAAAGCTACAAGAGCACATCGTTAAGAAAATCCTGGACTTCAATACTGATG
GGCTCGAAGACAAAAAAAATCTCCGGAGTTGGCTGGGTATTCTCAACTATG
CAAGGGCATACATACCAAACCTAGGAAATT

Appendix VII

PYMoV isolate from Pampadumpara panchayat

Pampadumpara (378 bp)

CTCGGACGGGGGTACACCGTGAAGTGTTCAGAGAAATGGACAACTGTTTCA
AAGGTATGGAAAATTTTATAGCTGTCTACATTGATGATATTCTGGTGTTCTCA
GAGAACATGAGGGACCATGCCCAACACTTAGTCGCAATGCTGGAAGTATGC
AAGAAGAATGGGCTTATTTTAAGCCCAACAAAAATGAAAATTGGGCTTGGA
ACTATTGATTTCCTGGGAGCAACTATTGGAAACAGCAAGGTAAAGCTACAA
GAGCACATCGTTAAGAAAATCCTGGACTTCAATACTGATGGGCTCGAAGAC
AAGAAGAATCTCCGGAGTTGGCTGGGTATTCTCAACTATGCAAGGGCATAC
ATACCAAACCTAGGAACAT

Appendix VIII

PYMoV isolate from Karunapuram panchayat

Karunapuram (378 bp)

CGTCGGTATACTCTGCATGTGTCAGAGAGGACGGACAACTGTTTTAAGGTAT
GGAAGATTTTATAGTTGTCTATATCGATGATATTCTGGTGTTCTCAGAAACAT
GAAGGACCATGCCCAACACTTAGTCGCAATGCTGGAAGTATGCAAGAAGAA
TGGGCTTATCCTAAGCCCAACAAAAATGAAGATTGGGCTTGGAACTATTGAC
TTCCTGGGAGCAACTATTGGAAACAGCAAGGTAAAATTGCAGGAGCACATC
GTCAAGAAAATTTTGGACTTCAATACTGATGGGCTCGAGGACAAGAAAAAT
CTCCGAAGTTGGCTAGGCATCCTCAACTATGCAAGAGCATACATACCAAACC
TAGGAACATCACTAGC

Appendix IX

PYMoV isolate from Ambalavayal panchayat

Ambalavayal (380bp)

CGGGGGTCGACATGTAGACATACTCCGGAGATTCTTCTTGTCTTCGAGCCCA
TCAGGTATTGAAGTCCAGGATTTTCTTAACGATGTGCTCTTGTAGCTTCACCT
TGCTGTTCCCAATGGTTGCTCCCAGGAAATCAATAGTTCCAAGCCCAATTTT
CATTTTTGTTGGGCTTAAGATAAGCCCATTCTTTTTGCATACTTCCAGCATTG
CAACTAAGTGTTGGGCATGGTCCCTCATGTTTTCTGAGAACACCAGAATATC
GTCAATGTAGACAACTATAAAATCTTCCATACCTTTGAAACAGTTGTCCATTT
TTCTTTGGAACACAGCAGGTGCATTTTTAAGCCCAAATGGCATCACTAGCCA
TTCATATAGAGTC

Appendix X

PYMoV isolate from Meenangadi panchayat

Meenangadi (424 bp)

CCGATCATTGGAGATGTCGACCACTCCGAAGATCTTTCTTGTCCTCGAGCCC
ATCAGTGTTGAAGTCCAGGATTTTCTTAACGATGTGCTCTTGGAGCTTTACC
TTGCTGTTTCCAATAGTTGCTCCCAGGAAATCAATAGTTCCAAGCCCAATTT
TCATTTTTGTTGGGCTTAGGATAAGCCCATTCTTCTTGCATACTTCCAGCATG
GCGACTAAGTGTTGGGCATGGTCCTTCATGTTTTCTGAGAACACCAAAATGT
CATCAATGTAGACAACTATAAAATCTTCCATACCTTTGAAACAGTTGTCCATT
TTTCTTTGGAACACAGCATTTTTTTTTTTTTAAGCCCAAATGGCACCACTAGC
CATGTATATTGAGCCTTTTTGGGGGGGGGAGGAACCCCACACCAAAAAAAA
AAAAAT

Appendix XI

PYMoV isolate from Poothadi panchayat

Poothadi (383 bp)

CGGGGGGGCCGTTGTTTACGACCACTCAGGAGATCTTCTTGTCTTCGAGCC
CATCAGTATGGAAGTCCAGGATTTTCTTAACGATGTGCTCTTGTAGCTTTACC
TTGCTGTTTCCAATAGTTGCTCCCAGGAAATCAATAGTTCCAAGCCCAATTT
TCATTTTTGTTGGGCTTAAGATAAGCCCATTCTTCTTGCATACTTCCAACATT
GCGACTAAGTGTTGGGCATGGTCCCTCATGTTTTCTGAGAACACCAGAATAT
CATCAATGTAGACAACTATGAAATCTTCCATACCTTTGAAACAGTTGTCCATT
TTTCTTTGGAACACAGCATGTGCATTTTTAAGCCCAAATGGCATCACTAGCC
GTGCATATTGAGTCCAT

Appendix XII

PYMoV isolate from Panamaram panchayat

Panamaram (511 bp)

CCGGGGCCGGGCTGTCGACGACTCCGGAGATCTTCTTGTCTTCGAGCCCAT
CAGTATTGAAGTCCAGGATTTTCTTAACGATGTGCTCTTGAAGCTTTACCTT
GCTGTTTCCAATAGTTGCTCCCAGGAAATCAATAGTTCCAAGCCCAATTTTC
ATTTTTGTTGGGCTTAAGATAAGCCCATTCTTCTTGCATACTTCCAGCATTGC
GACTAAGTGTTGGGCATGGTCCCTCATGTTTTCTGAGAACACCAGAATATCA
TCAATGTAGACAACTATAAAATCTTCCATACCTTTGAAACAGTTGTCCATTTT
TCTTTGGAACACAGCAGGTGCATTTTTAAGCCCAAATGGCATCACTAGCCTT
TCATATAGACCCACCTGGTTGGGTAAGTTACGAACCTATGGGAAAGGAGTCT
GTTAGCATTCATGAGGGACCAGGTTGAAAAGAATGAACACGTACATACAAA
ATGCTGAAATGTTGCTCTCTCAAAGTATGAATTAGGTCCACGG

Varietal screening of black pepper to *Cucumber mosaic virus* and *Piper yellow mottle virus* and their sero-molecular detection

by

ARYA M.

(2018-11-013)

Abstract of the thesis

Submitted in partial fulfilment 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

2020

ABSTRACT

Varietal screening of black pepper to *Cucumber mosaic virus* and *Piper yellow mottle virus* and their sero-molecular detection

The study entitled "Varietal screening of black pepper to *Cucumber mosaic virus* and *Piper yellow mottle virus* and their sero-molecular detection' was conducted at Department of Plant Pathology, College of Agriculture, Vellayani during 2018-2020 with the objectives to study the occurrence and sero-molecular detection of *Cucumber mosaic virus* (CMV) and *Piper yellow mottle virus* (PYMoV) infecting black pepper and to identify the varietal response to infection by the viruses.

As a part of the study, survey was conducted in four panchayats of Idukki (Erattiyar, Kattappana, Pampadumpara and Karunapuram) and Wayanad (Ambalavayal, Meenangadi, Poothadi and Panamaram) districts of Kerala. Plant samples showing the symptoms of viral infections were collected and severity of viral infections were assessed from farmer's field at the survey locations. Highest disease incidence (DI) was observed in Poothadi (45.50 %) of Wayanad and lowest in Karunapuram (21.62 %) of Idukki district. Highest vulnerability index (V.I.) was recorded in Panamaram (61) of Wayanad and lowest in Pampadumpara (32) of Idukki district. Mealy bugs were found associated with the virus infected plants in survey locations.

Different symptoms of viral infections observed in the field were general chlorosis, initial chlorotic specks progressing to severe mottling and curling, vein banding, vein clearing, leaf distortion and reduction in leaf size. The virus inoculum was maintained in insect proof greenhouse condition. In the field, high viral disease incidence was observed in Karimunda plants while Thekkan pepper grafted on *Piper colubrinum* did not express any symptom. Varietal screening with 15 varieties (KAU,

IISR and local) was undertaken by wedge grafting the infected scions showing the symptoms of both viruses (CMV and PYMoV) on to healthy black pepper plants. Among the varieties screened, Karimunda was identified as the most susceptible variety with highest V.I. (48).

Serological detection of CMV and PYMoV were confirmed using polyclonal antisera of *Cucumber mosaic virus* (CMV) and two monoclonal antibodies of *Banana streak virus* (BSV 1 and BSV 2; as PYMoV belongs to the same group of BSV) through DAS (Double Antibody Sandwich)-ELISA. Infection due to either of the two viruses and mixed infections by both viruses were detected in the leaf samples. Leaf curling was found associated with infection by PYMoV while leaf reduction and distortion in leaf size were found associated with CMV infection. The results were confirmed using polyclonal antibodies of CMV and *Sugarcane bacilliform virus* (SCBV; as PYMoV belongs to the same group of SCBV) through DIBA (Dot Immuno Binding Assay) in infected leaf samples.

The quality and quantity of isolated DNA by CTAB method was also assessed. PCR based molecular detection of PYMoV using ORF III specific primers yielded amplicons of approximately 400 bp. The amplified PCR products were subjected to sequencing and blast analysis. All the isolated sequences were found to be related to PYMoV. Phylogenetic studies revealed that isolates from Idukki and Wayanad grouped in separate clades.

The present study on viruses infecting black pepper indicated that infection of CMV and PYMoV either in pure form or as mixed infections was prevalent in cultivated areas of Idukki and Wayanad districts of Kerala. Serological techniques like DAS-ELISA and DIBA could be used for detection of these viruses along with molecular detection using coat protein specific primers. Varietal screening undertaken revealed that Karimunda was the most susceptible variety. The isolates of PYMoV from different locations of Idukki and Wayanad showed similarity in ORF III to other reported PYMoV sequences.

സംഗ്രഹം

കുരുമുളകിലെ വൈറസ് രോഗങ്ങൾക്കെതിരെ ഇനങ്ങളുടെ പ്രതികരണവും കുരുമുളകിലെ വൈറസ് ഹേതുക്കളുടെ സീറോ മോളിക്കുലാർ പഠനവും എന്ന വിഷയത്തിൽ പഠനം വെള്ളായണി കാർഷിക കോളജിലെ സസ്യരോഗ വിഭാഗത്തിൽ 2018-20 കാലയളവിൽ നടത്തുകയുണ്ടായി. പഠനത്തിൻ്റെ ഭാഗമായി കുരുമുളക് കൃഷി വ്യപകമായി നടത്തുന്ന ഇടുക്കി, വയനാട് ജില്ലകളിലെ 4 പഞ്ചായത്തുകളിൽ സർവേ നടത്തുകയുണ്ടായി.അതിനായി ഇടുക്കിയിലെ ഇരട്ടിയാർ, കട്ടപ്പന, പാമ്പാടുംപാറ, കരുണാപുരം എന്നീ പഞ്ചായത്തുകളും വയനാട് ജില്ലയിൽ നിന്നും അമ്പലവയൽ, മീനങ്ങാടി, പൂതാടി, പനമരം എന്നീ പഞ്ചായത്തുകളും സന്ദർശിച്ചു.വൈറസ് രോഗ ലക്ഷണം പ്രകടിപ്പിച്ച കുരുമുളക് വള്ളികൾ ശേഖരിക്കുകയും അവയുടെ രോഗ വ്യപനവും രോഗ തീവ്രതയും വിലയിരുത്തുകയുണ്ടായി.ഏറ്റവും കൂടുതൽ രോഗത്തിന്റെ വ്യപനം റിപ്പോർട്ട് ചെയ്തത് വയനാട്ടിലെ പൂതാടി പഞ്ചായത്തിലും (45.5 %) ഏറ്റവും കുറവ് ഇടുക്കിയിലെ കരുണാപുരം പഞ്ചായത്തിലും ആണ്.രോഗ വ്യപനത്തിന് ഹേതുക്കളായ മുഞ്ഞ, മീലിമൂട്ട തുടങ്ങിയവയുടെ സാന്നിധ്യവും വള്ളികളിൽ നിന്നും ശേഖരിച്ചു. കൊടികളിൽ കണ്ട പലതരം രോഗ ലക്ഷണങ്ങളും പഠിക്കുയുണ്ടായി.മഞ്ഞളിപ്പ്, കുരുടിപ്പ് തുടങ്ങിയവ ആണ് പ്രധാന രോഗ ലക്ഷണങ്ങൾ. വിവിധഇനങ്ങളുടെ രോഗ പ്രതിരോധ ശേഷി അളക്കുന്നതിനായി രോഗമുള്ള വള്ളി രോഗ രഹിതമായ

വള്ളികളിൽ ഗ്രാഫ്റ്റ് ചെയ്തു. കരിമുണ്ട എന്ന കൊടിയിനത്തിനാണ് പ്രതിരോധ ശേഷി ഏറ്റവും കുറവ് എന്ന് കണ്ടെത്തി. പുതിയ ഇലകളിൽ രോഗലക്ഷണങ്ങൾ പ്രകടമാക്കുന്ന ദിവസവും അവയുടെ തീവ്രതയും പഠിച്ചു.രോഗം നിജപ്പെടുത്തുന്നതിനായി സീറോ മോളിക്കുലാർ രീതികളായ എലിസ, ഡിബ, പി. സി. ആർ എന്നീ ടെസ്റ്റുകൾക്ക് വിധേയമാക്കി കൊണ്ട് വൈറസ് സാന്നിധ്യം സ്ഥിരികരിച്ചു. രോഗകാരിയായ പെപ്പർ യെല്ലോ മോട്ടൽ വൈറസിന്റെ ഒ.ആർ.എഫ് 3 ജനിതക മാത്രകളും ഇവയ്ക്ക് മറ്റ് വൈറസുകളുമായി താരതമ്യ പഠനവും (ഫൈലോജനി ട്രീ) നടത്തി.