# RPF-III

## PERFORMA FOR SUBMISSION OF FINAL REPORT OF RESEARCH PROJECTS

Part- I : General Information

**800 Project Code** : **Path. XII (813)**

8001 Institute Project Code No. : DBT-CP1

8002 ICAR Project Code No.

**801 Name of the Institute and Division**

8011 Name and address of Institute : Indian Institute of Spices Research, Calicut –673 012, Kerala

8012 Name of Division / Section : Division of Crop Protection

8013 Location of the Project : IISR, Calicut, Kerala, India

**802 Project Title** **:** Molecular characterization of viruses causing stunted disease in black pepper and development of PCR based methodology for their detection

**803 Priority Area** : Crop Protection

8031 Research Approach :

| Applied Res. | Basic Res. | Process or Tech. Dev. | Transfer of Tech. |
|---|---|---|---|
| ✓ | ✓ | ✓ | |

804 Specific Area : Identification & development of diagnostics against pathogen

**805 Duration of Project** : 3 years

8051 Date of start : 6th June 2003

8052 Date of Completion : 5th June 2006

**806 Total cost /Expenditure Incurred** : Rs **22,99,167**
(Give reasons for variation, if any from original estimated cost)

**807 Executive Summary**

One hundred sixty five isolates of stunted disease affected black pepper (*Piper nigrum*) plants were collected from different regions of Kerala and Karnataka during survey and were maintained under insect proof conditions. A few isolates of *P. longum* and *P. betle* showing virus like symptoms were also collected and maintained. Serological analysis of the above isolates using DAC-ELISA revealed the presence of

two viruses namely *Cucumber mosaic virus* (CMV) and a *Badnavirus* with the diseased vines. Based on the available sequence from GenBank, primers were designed for amplifying coat protein (CP) gene of CMV and RT-PCR was performed. A product of about 650 bp amplified from the infected sample was cloned into pPCR Script Amp (SK+) vector and sequenced. The sequenced region contained a single open reading frame of 657 bases encoding for a protein of 218 amino acids. CP gene of black pepper isolate of CMV was compared with corresponding gene of 25 distinct CMV isolates belonging to both the subgroups (subgroup I and II) from different hosts at nucleotide and amino acid levels. Sequence comparison and phylogenetic analysis revealed that CMV infecting black pepper belongs to subgroup IB. CP gene of CMV infecting *P. longum* and *P. betle* were amplified, cloned and sequenced. Pair wise comparison between them showed 100% sequence identities both at nucleotide and amino acid levels while sequence comparison and phylogenetic analysis with other 27 distinct CMV isolates showed that CMV infecting *P. longum* and *P. betle* belongs to subgroup IB. Further sequence analysis showed a very high level of sequence identity (>99%) both at nucleotide and amino acid level among all the four isolates of CMv infecting *Piper* spp.

A total of 11 primers were designed and used in PCR for the amplification of badnaviral genome, and of which two primer pairs were found to be successful in amplifying portion of ORF I and ORF III. The products corresponding to ORF I and III were cloned and sequenced. Comparison of the 694 bp sequences (corresponding to ORF-I) with other badnaviruses showed that the badnavirus infecting black pepper as close to the *Piper yellow mottle virus* (PYMV) (with an identity of 94.8 and 95.2% at the nucleotide and amino acid level respectively) followed by *Banana streak virus* (BSV). Sequence comparison of the 597 bases corresponding to ORF III with other badnaviruses showed high proximity of the black pepper isolate to the isolates of BSV. Thus based on sequence analyses of ORF I and III, it was concluded that *Badnavirus* infecting black pepper in India is a strain of PYMV. A portion of ORF I and ORF III from four isolates representing different geographical regions (Calicut, Idukki and Wynad districts of Kerala and Kodagu district of Karnataka) were cloned and sequenced. Analysis using ORF I sequences showed high variability (38.9 to 97.9%) among different isolates while OFR-III sequences were highly conserved among isolates.

A protocol was developed for the isolation of total RNA from infected black pepper to be used as template in RT-PCR. RT-PCR based detection methodology was developed for CMV wherein. Similarly, a reliable protocol for the extraction of total DNA from black pepper and PCR for the detection of PYMV was standardized. A sensitive protocol was developed for the isolation of total nucleic acids from black pepper. A single tube multiplex RTCR was standardized for the simultaneous detection of CMV and PYMV infecting black pepper.

**808 Key words** **:** stunted disease, identification, Cucumber mosaic virus, Piper yellow mottle virus, sequence analyses, detection, multiplex PCR.

**Part-II : Investigator Profile**

(Please identify clearly changes, if any in Project personnel)

**810 Principal Investigator :**

| | | |
|---|---|---|
| 8101 | Name | : Dr. A.Ishwara Bhat |
| 8102 | Designation | : Senior Scientist |
| 8103 | Division/ Section | : Crop Protection |
| 8104 | Location | : Indian Institute of Spices Research |
| 8105 | Institute Address | : Indian Institute of Spices Research<br>Calicut-673 012, Kerala, India |

**811 Co- Investigator:**

| | | |
|---|---|---|
| 8111 | Name | : Dr. R. Suseela Bhai |
| 8112 | Designation | : Senior Scientist |
| 8113 | Division/ Section | : Crop Protection |
| 8114 | Location | : Indian Institute of Spices Research |
| 8115 | Institute Address | : Calicut-673 012, Kerala, India |

## Par-III: Technical Details

**820 Introduction and objectives**

8201 Project Objectives:

i) Biological and molecular characterization of viruses associated with stunted disease in black pepper

ii) Determination of exact taxonomic status of these viruses

iii) Development of virus specific oligonucleotides for use in PCR for the specific detection and differentiation of viruses in field samples

iv) Standardization of multiplex PCR methodology for indexing planting material

**8202 Background information and importance of the projects**

Stunted disease of black pepper which was first observed at Neriamangalam, Idukki, Kerala in 1975 has now become a serious problem in all pepper growing regions of the country (Pailey et al., 1981). Although the disease is known to be of viral nature, the exact identification of the causal virus needed in depth investigations. Sarma *et al*. (2001) have reported *Cucumber mosaic virus* (CMV) as one of the causal

viruses based on host range, particle morphology and serology. In addition Bhat *et al.* (2003) reported the association of a *Badnavirus* serologically related to *Banana streak virus* and *Sugarcane bacilliform virus* with stunted disease of black pepper based on electron microscopy, mealybug transmission and serological relationships. However, detailed investigation on the molecular characterization of the viruses associated with black pepper stunted disease and their correct taxonomic identification in India is lacking. Disease of similar nature has also been reported from many other black pepper growing countries of the world. Association of a badnavirus, *Piper yellow mottle virus* (PYMV) was shown to be one of the causal viruses in the stunted disease affected black pepper vines in many south east Asian countries including Brazil, Malaysia, The Philippines, Sri Lanka and Thailand (Lockhart *et al*., 1997; Duarte *et al*., 2001; de Silva *et al*., 2002). At present there is no correct method of diagnosing diseased plants and hence the disease is fast spreading in the country through the use of infected cuttings for planting. So there is a need for the correct identification and molecular characterization of different viruses associated with stunted disease of black pepper in India. Thus under this project identification of the causal viruses associated with the disease, cloning and sequencing of associated viral genomes for their correct taxonomic identification was taken up. As black pepper is clonally propagated through stem cuttings major means of spread of viruses is through use of infected cuttings for planting. Hence project also came with protocols for the isolation of nucleic acid from black pepper and detection of the viruses thorough PCR. A procedure for simultaneous isolation of RNA and DNA from black pepper plant and multiplex reverse transcription polymerase chain reaction (mRT-PCR) for simultaneous detection of viruses associated with stunted disease was developed. This will help for the development of virus-free planting material certification programme in black pepper.

## 821 Project Technical Profile

8211 Technical programme
(Indicate briefly plan of procedure, techniques, instruments and special materials, organisms, special environments etc.)

Collection and maintenance of isolates
Serological analysis of isolates

Molecular characterization of *Cucumber mosaic virus* (CMV) infecting black pepper
Molecular characterization of CMV infecting *Piper longum* and *P. betle.*
Comparison of CMV isolates infecting *Piper* spp.
Molecular characterization of the *Badnavirus* infecting black pepper.
Comparison of *Piper yellow mottle virus* (PYMV) isolates infecting black pepper
Molecular characterization of *Badnavirus* infecting *Piper longum* and *P. betle*
Detection of CMV in black pepper
Detection of PYMV in black pepper
Development of multiplex RT-PCR for simultaneous detection of CMV and PYMV infecting black pepper

8212 Total man months involvement of component project workers

a) Scientific 18
b) Technical 72
c) Supporting 12

**822 Final Report on the Project**

Detailed report containing all relevant data with a summary of results (not exceeding 2-5 pages)

8221 Achievements in terms of targets fixed for each activity

**1. Collection of virus isolates**

Black pepper (*Piper nigrum* L.) gardens at Dakshina Kannada, Kodagu, Hassan, Udupi and Uttara Kannada districts of Karnataka and, Kasargod, Kannur, Idukki and Wynad districts of Kerala were surveyed for the incidence and collection of isolates of stunted disease. Very high incidence and intensity of the disease was noticed at Idukki and Wynad districts with many gardens showing 100% disease incidence. In general disease incidence and severity were more in Kerala than in Karnataka. A wide range of symptoms are observed on infected vines. Mosaic, mottling and small leaf conditions are most obvious symptoms for identifying the disease in the field. The symptoms on leaves include vein clearing, yellow specks distortion, reduction in size, mottling and mosaic, along with stunting of the whole plant (Fig. 1a, b). The infected vines produce short spikes with poor filling leading to yield reduction. In severe cases, the leaves become abnormally narrow and give a sickle shaped appearance. The internodes of vines become abnormally short leading to stunting of plants. Few of the diseased gardens especially at lower elevations showed bright yellow chlorotic mottling along veins, chlorosis, vein clearing, leaf distortion, reduced plant vigour and poor fruit set. Yield loss vary depending on the age at which vine gets infected. Yield loss can be as high as 89% if the planting material is infected at the time of planting. One hundred and sixty six virus isolates collected from different geographical were maintained under insect proof conditions through vegetative propagation for further studies.

**2. Serological analysis of the isolates**

All the collected isolates were subjected to serological analysis using direct antigen coated enzyme linked immunosorbent assay (DAC-ELISA) using antisera to badna-,

cucumo-, ilar-, poty- and tospo- viruses. Polyclonal antisera to different badna viruses namely, *Commelina yellow mottle virus* (ComYMV), *Banana streak virus* (BSV), *Rice tungro bacilliform virus* (RTBV) and *Sugarcane bacilliform virus* (SCBV) and, antiserum to a cucumovirus (*Cucumber mosaic virus*, CMV), potyvirus (*Potato virus Y*, PVY), ilarvirus (*Tobacco streak virus*, TSV) and tospovirus (*Groundnut bud necrosis virus*, GBNV) were used in tests. The assay was performed on polystyrene plate (Co-Star). Infected and healthy leaf tissues were triturated at 1:5 dilution in coating buffer containing 2% polyvinyl pyrrolidone (PVP, MW 40,000). These extracts were centrifuged briefly and the supernatant was placed into three wells per sample and incubated at 37°C for 1h. Polyclonal rabbit antisera were used at 1:1000 except for CMV which was used at 1:100 (as per supplier's instructions), and antirabbit immunoglobulin-alkaline phosphatase conjugate (Sigma St. Luis, USA) was used at 1:20,000. Absorption values at 405 nm were recorded using a microplate reader (Bio-Tek Instruments), 1 h after adding the substrate, *p*-nitro phenyl phosphate (0.5mg/ml of substrate buffer).

Results of DAC-ELISA showed that none of the samples reacted with GBNV, PVY and TSV anstisera, suggesting the lack of association of a tospo-, poty- or ilar-viruses with the disease. Positive reaction was observed with CMV and two of the badnavirus antisera tested. Among the antisera to different badnaviruses tested, none of the samples reacted with RTBV and ComYMV antisera while all the samples reacted with BSV and SCBV antisera suggesting the association of a badnavirus serologically related to BSV or SCBV. Majority of the isolates showed positive reaction with only either CMV or badnavirus (BSV & SCBV) while a few of the isolates showed positive reaction with both CMV and badnavirus (BSV & SCBV). Varying O.D values obtained with different samples indicate varying concentration of viruses in the diseased plant. Of the two badnaviruses, majority of the samples reacted more strongly with BSV antiserum suggesting close antigenic relationship between black pepper badnavirus and BSV. These results indicate possible involvement of two viruses belonging to genera, badna and cucumo in the stunted disease of black pepper occurring in India. Based on these results, using CLUSTAL W multiple alignment program, the conserved region in the badnavirus and *Cucumber mosaic virus* have been located by aligning all known sequences available from the GenBank. Oligonucleotide primer pairs corresponding to both badna and CMV have been synthesized for use in polymerase chain reaction (PCR) to amplify viral sequences.

## 3. Molecular characterization of *Cucumber mosaic virus* (CMV) infecting black pepper

### 3. 1. Amplification of Coat Protein gene of CMV

ELISA positive isolates were used in this study. Total RNAs from the sample extracted using Nucleospin RNA plant Kit (Macherey-Nagel, Duren Germany) were used template were used as a template for reverse transcription. RT and PCR were performed in the same tube without any buffer changes in between. The primer pair designed from the CP gene sequences of CMV was used in the reaction to prime the amplification. The genome sense primer 5'ATGGACAAATCTGAATCAAC 3' derived from the beginning of first 20 bases of the coding region and the genome antisense primer 5'TCAAACTGGGA GCACCC 3' represents the last 17 bases of the CP gene of CMV. The reverse transcription PCR was carried out with the following

components in a total of (100 µl) reaction volume, containing 30 pmole each of the primers, 20 units Ribonuclease inhibitor (Genei, Bangalore, India), 10 units AMV reverse transcriptase (Finnzymes OY, Finland), 2.5 units *Taq* Polymerase (Genei, Bangalore, India), 1x PCR buffer (Genei, Bangalore, India), 10 mM Dithiothreitol, (Genei, Bangalore, India) and 10 µM each of the dNTPs (Finnzymes OY, Finland). PCR mix (27 µl) containing the above components was added to the tubes containing the template RNA (73 µl). Amplification was performed in an automated thermal cycler (Eppendorf Master Cycler Gradient) and the programme consisted of one cycle at 42° C for 45 min for cDNA synthesis followed by 40 cycle reaction profile involving 30 s of denaturation at 94° C, 1 min of annealing at 50° C, and 1 min of extension at 72° C and a single cycle of final extension at 72° C for 10 min.

### 3.2. Analysis of the RT-PCR products

The RT-PCR products were analyzed by 1% agarose gel containing ethidium bromide. Samples were loaded along with 500bp DNA ladder. Tris-Acetate EDTA (TAE) buffer was used in the electrophoresis. The DNA was visualized using a UV transilluminator and a gel documentation apparatus. RT-PCR was successful in amplifying the CMV coat protein gene from black pepper samples. A PCR product of expected size (ca. 650 bp) was observed only in infected samples and not with healthy black pepper samples (Fig.2).

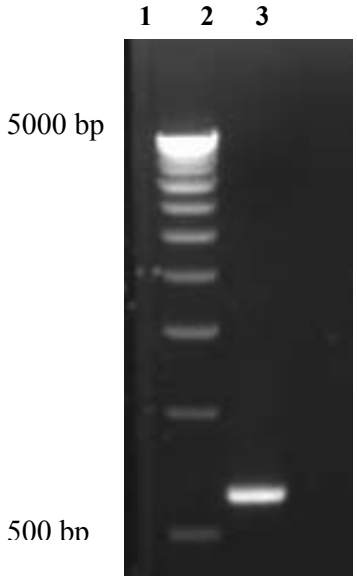


**Fig. 2. Analysis of RT-PCR products. Lane 1: 500bp DNA ladder, Lane 2: CMV infected black pepper, Lane 3: Healthy black pepper**

### 3.3. Cloning

The PCR product was purified using Strata Prep PCR purification Kit (Stratagene, La Jolla, CA, USA) and the purified products later on subjected to polishing with P*fu* polymerase and dNTP mix. The resultant product was then cloned in to pPCR Script Amp (SK+) vector using pPCR Script Amp (SK+) cloning vector Kit. The *E.coli* cells (DH 5∝) were transformed with the ligated product using standard procedures as described by Sambrook and Russel (2001). True clones were identified by restriction

digestion of DNA isolated for each of the clones and also through PCR using CMV CP specific primers.

### 3.4. Sequencing and sequence analyses

Selected positive clones were sequenced at the automated facility available at Avestha Gen Graine Technologies Pvt. Ltd, Bangalore. Sequences were initially compiled using Seqaid programme (Rhoads and Roufa, 1989). The sequenced region contained a single open reading frame of 657 bases that could potentially code for a protein of 218 amino acids (Fig. 3).

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ATGGACAAATCTGAATCAACCAGTGCTGGTCGCAACCGTCGACGTCGTCCGCGTCGCGGTTCCCGCTCCG      70
M  D  K  S  E  S  T  S  A  G  R  N  R  R  R  R  P  R  R  G  S  R  S  A      24

CTTCCTCCTCCGCGGATGCTACATTTAGAGTCCTGTCGCAACAGCTTTCGCGACTTAATAAGACGTTAGC     140
   S  S  S  A  D  A  T  F  R  V  L  S  Q  Q  L  S  R  L  N  K  T  L  A       47

AGCTGGTCGTCCTACTATTAACCACCCAACCTTTGTGGGTAGTGAGCGCTGTAAACCTGGATACACGTTC     210
 A  G  R  P  T  I  N  H  P  T  F  V  G  S  E  R  C  K  P  G  Y  T  F       70

ACATCTATTACCCTGAAGCCTCCGAAAATAGACAAAGGGTCTTATTATGGTAAAAGGTTGTTACTTCCTG     280
T  S  I  T  L  K  P  P  K  I  D  K  G  S  Y  Y  G  K  R  L  L  L  P  D      94

ATTCAGTCACTGAGTTCGATAAGAAGCTTGTTTCGCGCATTCAAATTCGAGTTAATCCTTTGCCGAAATT     350
   S  V  T  E  F  D  K  K  L  V  S  R  I  Q  I  R  V  N  P  L  P  K  F     117

TGATTCTACTGTGTGGGTGACAGTCCGTAAAGTTCCTGCCTCCTCGGACTTGTCCGTTTCCGCCATCTCT     420
 D  S  T  V  W  V  T  V  R  K  V  P  A  S  S  D  L  S  V  S  A  I  S      140

GCCATGTTTGCGGACGGAGCCTCACCAGTACTGGTTTATCAGTATGCCGCGTCCGGAGTCCAAGCCAACA     490
A  M  F  A  D  G  A  S  P  V  L  V  Y  Q  Y  A  A  S  G  V  Q  A  N  N     164

ATAAATTGTTGTACGATCTTTCGGTGATGCGCGCTGATATTGGTGACATGAGAAAGTACGCCGTGCTCGT     560
   K  L  L  Y  D  L  S  V  M  R  A  D  I  G  D  M  R  K  Y  A  V  L  V     187

GTATTCAAAAGACGATGCGCTCGAGACGGATGAACTAGTACTTCATGTCGACATTGAGCACCAACGTATT     630
 Y  S  K  D  D  A  L  E  T  D  E  L  V  L  H  V  D  I  E  H  Q  R  I      210

CCCACTTCTGGGGTGCTCCCAGTTTGA     657
P  T  S  G  V  L  P  V  <       218
```

**Fig.3. Nucleotide and deduced amino acid sequence of coat protein gene of CMV infecting black pepper in India.**

The coat protein gene of the black pepper isolate was compared with corresponding gene of CMV isolates belonging to both the subgroups of CMV from different hosts and region including India from GenBank (Benson *et al.,* 1999) (Table 1). at the nucleotide and amino acid level. The BLAST programme (Altschul et al., 1999) was used to download related sequences from Genbank. Multiple sequence alignments were made using CLUSTAL X (Thompson et al., 1997) and sequence phylograms were constructed using PHYLIP package (Bootstrap analysis with 1000 replicates), and unrooted trees were generated using TREEVIEW software (Page, 1996). The coat protein gene of black pepper isolate of CMV shared 89 to 95% identity with various isolates of CMV belonging to subgroup I while per cent identity ranged from 75 to 76 with various isolates of CMV in the subgroup II. Similarly, comparison of amino acid sequences of the coat proteins revealed that coat protein of black pepper isolate shared 92 to 99% identitywith members of subgroup I and from 77 to 79% identity with members of subgroup II. An identity of >95% were observed

**Table 1: Source of CMV coat protein gene sequence used for comparisons**

| CMV Subgroup | Country | Designation | Gen Bank Accession No. |
|---|---|---|---|
| Subgroup I | China | PI | AJ006988 |
| | China | ChCu | X65017 |
| | China | SD | AB008777 |
| | India | IN-Am | AF198622 |
| | India | IN-Ba | AY125575 |
| | India | IN-Di | AF281864 |
| | India | IN-Hb | AF350450 |
| | India | PhyM | X89652 |
| | Israel | Ban | U43888 |
| | Japan | D8 | AB004781 |
| | Japan | Leg | D16405 |
| | Japan | N | D28486 |
| | Japan | Pepo | D43800 |
| | Japan | C7-2 | D42079 |
| | Korea | Kor | L36251 |
| | Korea | ABI | L36525 |
| | Taiwan | M48 | D49496 |
| | Taiwan | NT9 | D28780 |
| Subgroup II | Australia | Q | M21464 |
| | Hungary | Irk | L15336 |
| | Japan | M2 | AB006813 |
| | Scotland | Kin | Z12818 |
| | South Africa | S | AF063610 |
| | United States | Ls | AF127976 |
| | United States | WL | D00463 |
| | India | In-Li | AJ585086 |
| | India | IN-Pn | AY545924 (present study) |
| | India | IN-Pb | AY690620 (present study) |
| | India | N-Pl | AY690621 (present study) |

with the available coat protein gene sequences of CMV isolates from India both at nucleotide and amino acid level. The sequence homology of coat protein gene ranges between 96.3% and 99.5% within a subgroup and between 76.0 and 77.5% for isolates of different subgroups. Homologies in coat protein amino acid sequences are 94.0 to 99.2% and 79.5% to 83.2% respectively (Palukaitis *et al*., 1992). In accordance with this the black pepper isolate tested in this study belong to subgroup I. Cluster dendrogram revealed that black pepper isolate was most closely related to members of subgroup I isolates (Fig. 4). In general, coat protein amino acid sequence identity among subgroup I isolates of the same geographic area is much higher than

that between isolates of different area. Thus phylogenetic analysis clearly showed that CMV infecting black pepper belongs to subgroup IB (Fig. 4).

**4. Molecular characterization of CMV infecting *Piper longum* and *P. betle.***

Betel vine (*Piper betle* L.*)* and Indian long pepper (*Piper longum* L.) are two important cultivated species of the genus *Piper* next to black pepper (*P. nigrum* L.). The viral like symptoms were characterized by the mosaic, mottling and reduction in leaf size in *P. betle* whereas mosaic, blisters, dark green patches and leaf distortion were observed in *P. longum*. Serological analysis of *P. betle* and *P. longum* isolates indicated the presence of two viruses namely *Cucumber mosaic virus* (CMV) and a *Badnavirus* with the diseased plants either as single or mixed infections.

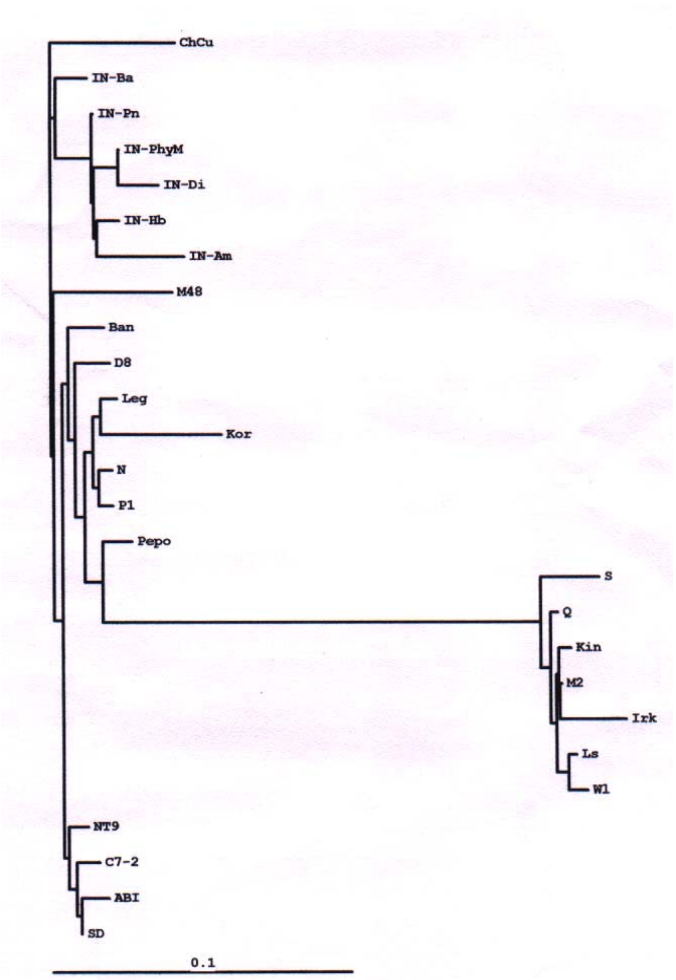


**Fig. 4. Cluster dendrogram illustrating phylogenetic relationships based on the multiple alignments of the coat protein amino acid sequences of 25 distinct isolates of CMV from various hosts and black pepper isolate of CMV (In-Pn). Designations given to each of the isolates is listed in Table 1.**

sequenced by chain termination at the automated DNA sequencing facility at the Avestha GenGraine Technologies Pvt Ltd., Bangalore. The sequenced region in both the cases contained a single open reading frame of 657 bases that could potentially code for a protein of 218 amino acids.

### 4.2. Sequence analyses

Pair wise comparison showed that the CP gene sequence of both the isolates of CMV from betel vine and Indian long pepper were highly conserved with 100% sequence identities both at nucleotide and amino acid levels (Fig. 6). The sequences of the CP gene of both the isolates were compared with those of other CMV isolates belonging to subgroup I and II from India, and a few representative isolates from other parts of the world (Table 1). CMV isolates of both *P. longum* and *P. betle* showed 93-97% and 95-99% identity at nucleotide and amino acid level, respectively, with CMV isolates belonging to subgroup I, while an identity of 71% and 79% was observed with only one available CMV isolate infecting *Lilium* belonging to subgroup II, from India (Fig. 6). An identity of 89-94% and 93-99% with

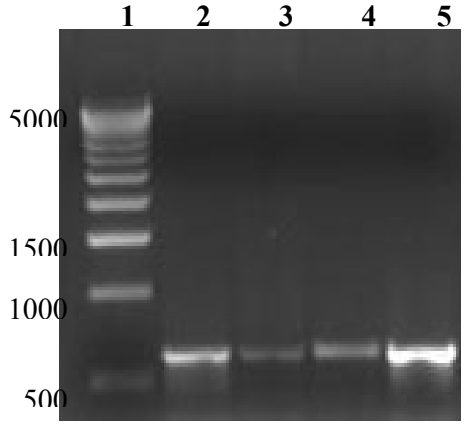


**Fig. 5. Analysis of RT-PCR product of coat protein (CP) gene of CMV from Indian long pepper and betelvine on agarose gel. Lane 1: 500bp DNA ladder and numbers on the left indicate the molecular weight in bp; Lane 2 & 3: Infected Indian long pepper, Lane 4 & 5: Infected betel vine**

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IN-Pb           MDKSESTSAGRN-RRRRPRRGSRSASSSADATFRVLSQQLSRLNKTLAAGRPTINHPTFV
IN-Pl           ************-**************************************************
IN-Ba           ************-**************************************************
IN-PhyM         ************-******************L***************************
IN-Di           ************-******************L***************************
IN-Pn           ************-**************************************************
IN-Hb           ************-****L*****************************************
IN-Am           ************-***HS*****************************************
In-Li           ****G*PN*S*TS*************G-***GL*A*T**MLK**R***I****LI**S**

IN-Pb           GSERCKPGYTFTSITLKPPKIDRGSYYGKRLLLPDSVTEFDKKLVSRIQIRVNPLPKFDS
IN-Pl           ************************************************************
IN-Ba           *****R*******************************************************
IN-PhyM         *********************K*************************V**********
IN-Di           *********************K*******W******************V**********
IN-Pn           *********************K************************************
IN-Hb           *********************K************************************
IN-Am           *****N**********R*****K************************************
In-Li           ***SGN**********N**EFEK***F*R**S******DY***********I********

IN-Pb           TVWVTVRKVPASSDLSVAAISAMFADGASPVLVYQYAASGVQANNKLLYDLSAMRADIGD
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**Fig. 6. Multiple alignment of coat protein amino acid sequences of *Cucumber mosaic virus (*CMV) isolates from India. Sequences for comparison were obtained from GenBank. The alignment was generated by Clustal W. Asterisk indicates identity and dash indicates deletion at a given position. Designations given to each isolate is given in Table 1**

selected CMV isolates from other parts of the world, at the nucleotide and amino acid levels, respectively, were observed with subgroup I isolates, while it ranged from 75-76 % and 77-79% with isolates belonging to subgroup II. The results of multiple alignments based on amino acid sequences of CP gene used to generate a phylogram clearly showed the closeness of *P. betle* and *P.longum* isolates of CMV with members of subgroup IB.

### 5.0. Comparison of CMV isolates infecting *Piper* spp.

In order to understand the diversity of the CMV infecting different *Piper* spp., coat protein gene of two isolates of CMV infecting black pepper from Calicut and Kasargod districts of Kerala and one isolate each infecting betel vine (*P. betle*) (Kasaragod, Kerala) and Indian long pepper (*P. longum*) (Calicut, Kerala) were cloned, sequenced and compared. Sequence analysis showed a very high level of sequence identity both at nucleotide and amino acid level among all the four isolates. The clustal X generated multiple alignment based on the deduced amino acid sequences of the CP gene of the isolates is presented in the Figure 7. The multiple alignments revealed a high degree of conservation (>99%) among CMV isolates tested. One amino acid deletion was observed in the Kasaragod isolate of CMV. Two isolates of CMV from black pepper differed at three positions. At the 82$^{nd}$ amino acid position lysine in Calicut isolate was replaced by glutamine in Kasaragod isolate, at the position 137, serine in Calicut isolate was replaced by alanine in Kasaragod isolate. Similarly at 192$^{nd}$ position valine in Calicut isolate was replaced by alanine in Kasraragod isolate.

```
PN-CLT      VWVTVRKVPASSDLSVSAISAMFADGASPVLVYQYAASGVQANNKLLYDLSVMRADIGDM
PN-KSD      ****************A**********************************A********
PB-KSD      ****************A**********************************A********
PL CLT      ****************A**********************************A********


PN-CLT      RKYAVLVYSKDDALETDELVLHVDIEHQRIPTSGVLPV
PN-KSD      ************************************
PB-KSD      ************************************
PL-CLT      ************************************
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**Fig. 7. Multiple alignment of deduced coat protein amino acid sequences of *Cucumber mosaic virus (*CMV) isolates of *Piper* spp. The alignment was generated by Clustal X. Asterisk indicates identity and dash indicates deletion at a given position. Designations: PN-CLT, Black pepper isolate from Calicut; PN-KSD, Black pepper isolate from Kasargod; PB-KSD, Betel vine isolate from Kasaragod; PL-CLT, Indian long pepper isolate from Calicut.**

## 6.0. Molecular characterization of the *Badnavirus* infecting black pepper.

### 6.1 Primer designing

In order to amplify the genome of *Badnavirus* infecting black pepper, oligonucleotide degenerate primer pairs were designed either based on multiple alignments of badnaviral sequences available in the GenBank or using the sequences of primers available in the literature. A total of 12 primers were designed and synthesized (Table 2).

**Table. 2. Degenerate primers designed for the amplification of *Badnavirus* infecting black pepper.**

| Oligo # | Primer Sequence (5'----3') | Region |
|---|---|---|
| AIB3 | TAC/T ATA/T/C GAC/T GAC/T ATA/T/C C/TT | Badna conserved region in RT, Forward primer (Lockhart *et* a*l*., 1997) |
| AIB4 | CCC CAT A/GCA A/C/G/TC CA/GT CA/G/C/T GTC/T TC | Badna conserved region in Rnase H domain, Reverse primer (Lockhart *et al.,* 1997) |
| AIB5 | ATA/C/T ATA/C/T ATA/C/T GAA/G ACC/T GA | Badna conserved region in Rnase H domain, Forward primer (Lockhart *et al* 1997) |
| AIB6 | A/CC/TA/C A/TA/T/G/CG CTC TGA TAC CA | Badna conserved region in tRNA binding site, Reverse primer (Lockhart *et al,* 1997) |
| AIB10 | CTC CTT CAT CTC CTC AAG AAG CCT | PYMV ORF I, Forward primer (de Silva *et al,* 2002) |
| AIB21 | CCA AAG CTC TGA TAG CAG AC | PYMV ORF I, Reverse primer (de Silva *et al,* 2002) |
| AIB24 | AAG TTT/C GAT/C C/TTG/A AAG/A A/TG/CT/C/G GG | Badna ORF III Forward primer (Based on multiple alignment of available badnaviral sequences) |

| | | |
|---|---|---|
| AIB25 | CTT/A/G/C TAT/C GAA TGG T/CTA/T GTG/A/T ATG CC | Badna ORF III Forward primer (Based on multiple alignment of available badnaviral sequences) |
| AIB26 | TA/GT TC/TC AA/GA/C GA/GA AAA/G ATG GA | Badna ORF III Forward primer (Based on multiple alignment of available badnaviral sequences) |
| AIB27 | CCA T/CTT G/ACA G/TAC/T T/GG/CC T/A/CC CCC | Badna ORF III, Reverse primer (Based on multiple alignment of available badnaviral sequences) |
| AIB28 | AAT/C T/GT/AG/C/T CCG/A CTT/A GCA/G TA | Badna ORF III Reverse primer (Based on multiple alignment of available badnaviral sequences) |
| AIB29 | TTC/T CCT/G/A TC/TG/A/T TGT GC/T TCA/G AT | Badna ORF III, Reverse primer (Based on multiple alignment of available badnaviral sequences) |

### 6.2 Amplification, cloning and sequencing

Total DNA from infected black pepper (Calicut isolate) was extracted using a slightly modified protocol described by de Silva *et al* (2002) except for the second extraction step which was carried out using equal volume of 10% CTAB (instead of 0.1 volume). Of the different primer combinations tested, two primer pairs were found successful in amplifying the respective regions from ORF I and ORF III of the *Badnavirus* infecting black pepper. The primer pair AIB 10 and AIB 21, amplified a fragment of ~700 bp from the ORF I (Fig. 8), whereas the primers AIB 25 and AIB 27

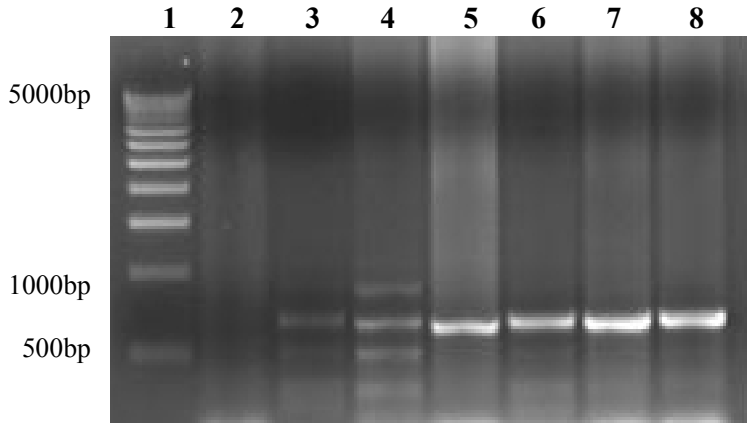


**Fig.8. Analysis of PCR products from ORF I of black pepper badnavirus on agarose gel. Lane 1: 500bp DNA ladder and numbers on the left indicates the molecular weight in bp, Lane 2: healthy black pepper, Lanes: 3-8: infected black pepper samples.**

**Table 3. Badnaviruses and their accession numbers used for comparisons**

| Sl.No. | Badnaviruses | GenBank Acc.No: | Designation |
|---|---|---|---|
| 1 | *Piper yellow mottle virus* | AJ626981 | PYMV |
| 2 | *Banana streak virus* | NC003381 | BSV-A |
| 3 | *Banana streak virus* | AY493509 | BSV-B |
| 4 | *Banana streak virus* | AY750155 | BSV-C |
| 5 | *Banana streak virus* | AY805074 | BSV-D |
| 6 | *Banana streak virus* | NC007003 | BSV-E |
| 7 | *Sugarcane bacilliform virus* | NC003031 | SCBV-A |
| 8 | *Sugarcane bacilliform virus* | M89923 | SCBV-B |
| 9 | *Cacao swollen shoot virus* | AJ534983 | CSSV-A |
| 10 | *Cacao swollen shoot virus* | NC001574 | CSSV-B |
| 11 | *Cacao swollen shoot virus* | AJ609019 | CSSV-D |
| 12 | *Cacao swollen shoot virus* | AJ609020 | CSSV-E |
| 13 | *Cacao swollen shoot virus* | AJ781003 | CSSV-F |
| 14 | *Kalanchoe top spotting virus* | NC004540 | KTSV-A |
| 15 | *Commelina yellow mottle virus* | NC001343 | CoYMV-A |
| 16 | *Citrus yellow mosaic virus* | NC003382 | CYMV-B |
| 17 | *Dioscorea alata bacilliform virus* | X94576 | DABV-H |
| 18 | *Rice tungro bacilliform virus* | AF113832 | RTBV |
| | | | |
| 19 | Badnavirus from black pepper | This study | PYMV-CLT |
| Out groups | | | |
| 20 | *Cauliflower mosaic virus* | AF140604 | CaMV-A |
| 21 | *Figwort mosaic virus* | NC003554 | FMV |

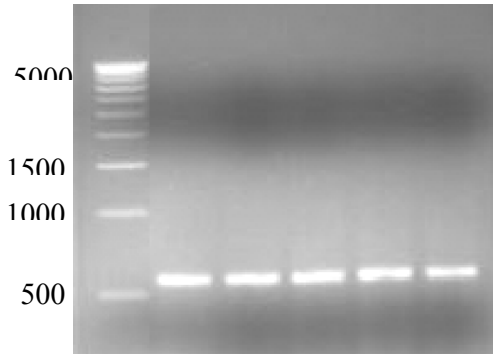


**Fig. 9. Analysis of PCR products from ORF III region of badnavirus from black pepper. Lane 1: 500bp DNA ladder and numbers on the left indicate the molecular weight in bp; Lanes 2-5 Infected black pepper plants**

### 6.3 Sequence comparison

**ORF-I**

Comparison of the 694 bp sequences (corresponding to ORF-I) with other badnaviruses (Table 3) showed that the *Badnavirus* infecting black pepper as close to the *Piper yellow mottle virus* (PYMV), with an identity of 94.8 and 95.2% at the nucleotide and amino acid level respectively. The identity with other badnaviruses ranged from 13.8 to 22.4% at the nucleotide level and 4 to 10.1% at the amino acid level. The cluster dendrogram also showed the high proximity of the black pepper isolate with PYMV reported from Southeast Asian countries, which formed a separate cluster (Fig. 10). Based on this, it was concluded that *Badnavirus* infecting black pepper in India is a strain of PYMV.

**ORF-III**

Sequence comparison of ORF III of Calicut isolate of PYMV infecting black pepper with other badnaviruses showed highly variable nature of the badnaviruses. Sequence of PYMV from other south East Asian counties was not available for comparison. Among the various badnaviruses used for comparisons, the black pepper isolate showed proximity to the isolates of *Banana streak virus* (BSV), with an identity ranging from 27.5 to 58.2% at the nucleic acid level and 63.2 to 67.7% at the amino acid level, followed by *Kalachoe top spotting virus* (KTSV) with an identity of 55.1 and 63.8%, *Sugarcane bacilliform virus* (SCBV) with an identity of 51-54.3 and 57.6 to 59.8% at nucleotide and amino acid levels respectively. The cluster dendrogram also indicated the same trend as many BSV isolates were clustered close to the PYMV isolates from black pepper (Fig. 11). The dendrogram showed close clustering of all CSSV isolates. BSV-D isolate showed distant relationship with rest of the BSV isolates.

### 7.0 Comparison of *Piper yellow mottle virus* (PYMV) isolates infecting black pepper

Badnaviruses in general, possess highly variable genomes at inter and intra specific levels. There have been reports on the genetic diversity of many badnaviruses within the species, as in the case of *Banana streak virus*, *Cacao swollen shoot virus*, *Taro bacilliform virus* etc. In view of this to understand the variability existing in *Piper yellow*
*mottle virus* (PYMV) infecting black pepper, a portion of ORF I and ORF III from four isolates representing different geographical regions (Calicut, Idukki and Wynad districts of Kerala and Kodagu district of Karnataka) were cloned and sequenced.

### 7.1 Variability analysis using ORF I sequences

The cloned ORF I region contained 694 nucleotides. The nucleotide sequence was used to generate multiple alignment using clustal X. The ORF I sequence of PYMV infecting black pepper in Sri Lanka was also used for comparison. Among the isolates, Calicut and Idukki showed high identity (96.4 and 97.9%) with the PYMV reported to infect black pepper in Sri Lanka whereas the Wyanad and Kodagu isolates showed low identity (38.9 and 44.0%) with PYMV. The cluster dendrogram based on ORF 1 sequences of different isolates of PYMV infecting black pepper is depicted in Fig.12. The dendrogram showed closeness of Calicut and Idukki isolates from Kerala

with that of PYMV from Sri Lanka while Kasaragod and Wyanad isolates were distantly related with other isolates and clustered separately (Fig. 12).

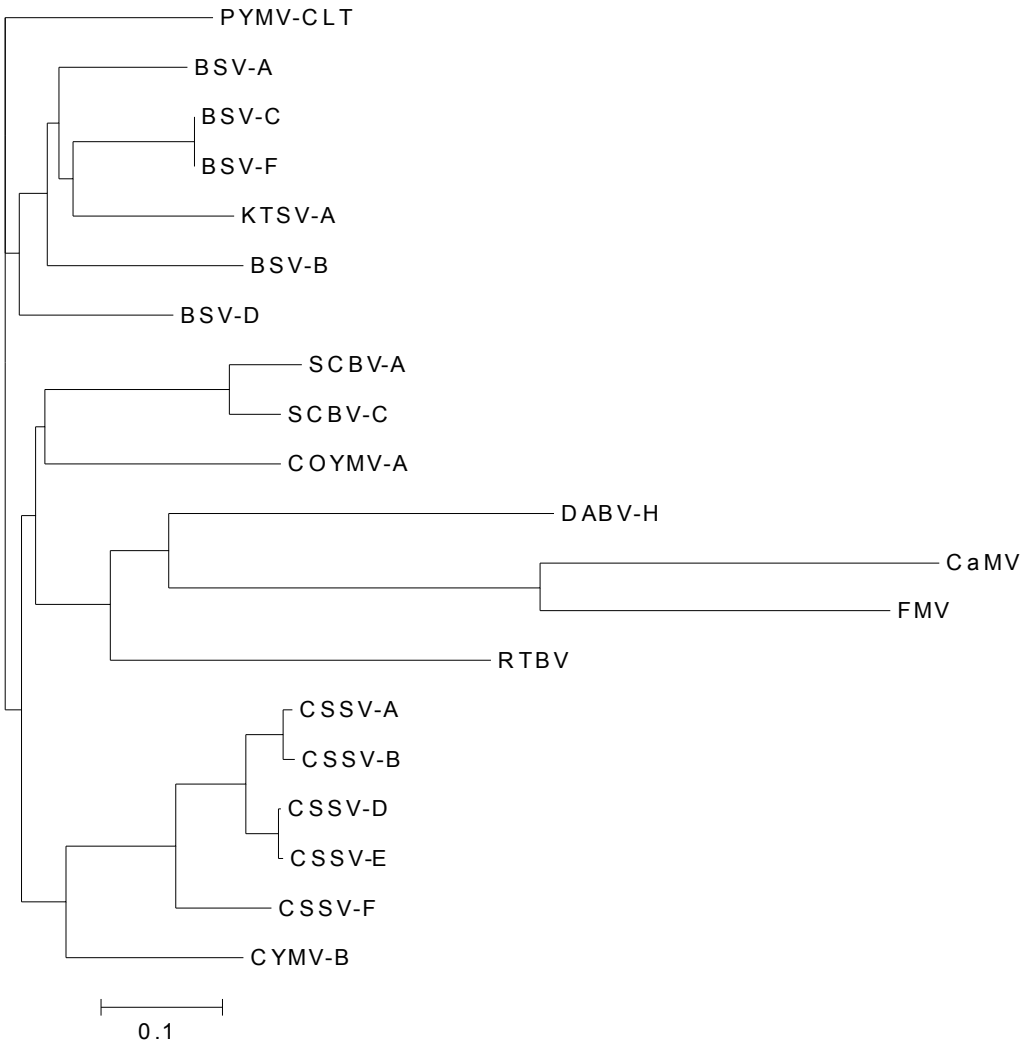


**Fig. 10. Cluster dendrogram illustrating phylogenetic relationships based on the multiple alignments of the ORF I nucleotide sequences of 16 distinct badnavirus isolates and black pepper isolate (PYMV-CLT). Designations used for each of the isolates are listed in Table 3.**

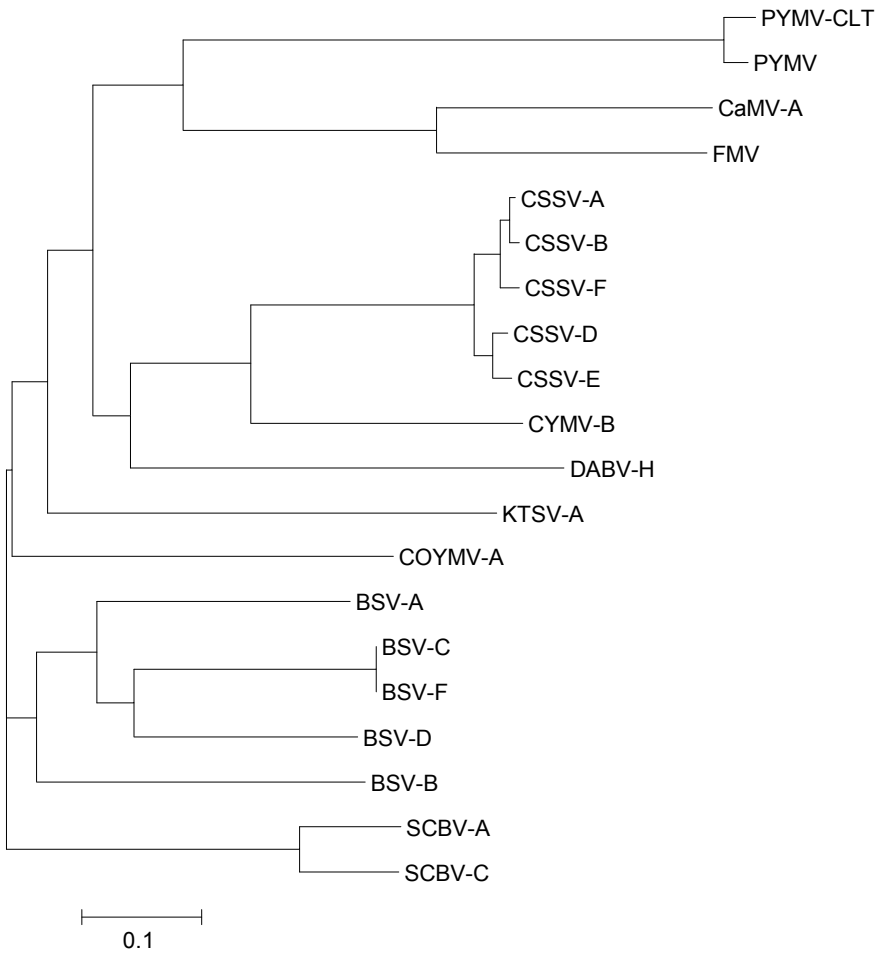


**Fig. 11. Cluster dendrogram illustrating phylogenetic relationships based on the multiple alignments of the ORF III amino acid sequences of 19 distinct badnavirus isolates and black pepper isolate (PYMV-CLT). The designations used for each isolate is given in Table 3.**

### 7.2 Variability analysis using ORF III sequences.

The portion of PYMV ORF III cloned contained ~597 nucleotides. The comparative study of this region among four isolates of black pepper showed high level of conservation at the nucleotide level (95.1 to 97.8%). At the amino acid level the identity ranged from 95.0 to 97.9%. Kodagu isolate was found to be distinct compared to other isolates (Fig. 13). Thus in general among the ORF I and III sequences compared, ORF III showed a high level of identitiy (>95%) while identity among isolates of PYMV in ORF I region was low

## 8.0. Molecular characterization of the *Badnavirus* infecting *P. betle* and *P. longum*

Primer pairs designed based on ORF I and ORF III regions of PYMV infecting black pepper in India were used to prime the reaction. Primers AIB 35 and AIB 36 specific for amplifying a portion of ORF I and primers AIB 25 and AIB 27 specific for amplifying a portion of ORF III were used. Total DNA isolated from

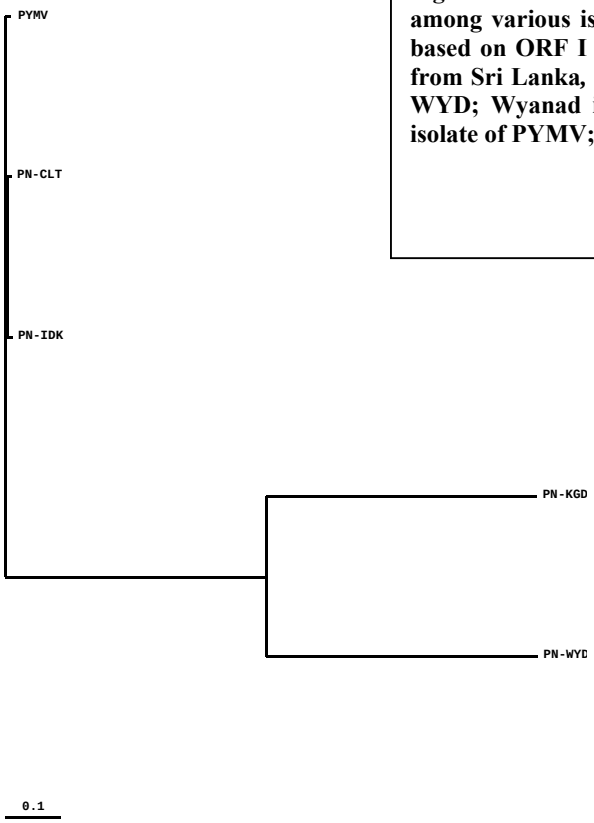


**Fig. 12 Cluster dendrogram revealing the relationship among various isolates of PYMV infecting black pepper based on ORF I sequence. Designations: PYMV, PYMV from Sri Lanka, PN-CLT, Calicut isolate of PYMV; PN-WYD; Wyanad isolate of PYMV; PN-KSD, Kasaragod isolate of PYMV; PN-IDK, Idukki isolate of PYMV.**

```
PYMV-CLT            -LYEWLVMPFGLKNAPAVFQRKMDNCFKGMEDFIAVYIDDILVFSENMRDHAQHLVAMLE
PYMV -WYD           --***F**************************************************
PYMV -IDK           -L*****************************************************
PYMV -KDG           ************************************************************

PYMV -CLT           VCKKNGLILSPTKMKIGLGTIDFLGATIGNSKVKLQEHIVKKILDFNTDGLEDKKNLRSW
PYMV -WYD           ************************************D***********************
PYMV -IDK           ************************************************************
PYMV -KDG           ************************************I***********************

PYMV -CLT           LGILNYARAYIPNLGRILGPLYAKVSPTGERKMNQQDWSIVAQIKKIIQELPELELPPED
PYMV -WYD           ************************************************************
PYMV -IDK           ************************************G***********************
PYMV -KDG           ********T**************I**********R**G***********************

PYMV -CLT           CCIVIKTDGCMSGWGAICKW
PYMV -WYD           *****E*********S****
PYMV -IDK           *****E**********V***
PYMV -KDG           *****E**************
```

**Fig.13. Multiple alignment of deduced amino acid sequences of a portion of ORF III of *Piper yellow mottle virus* isolates from black pepper. The alignment was generated by Clustal X. Asterisk indicates identity and dash indicates deletion at a given position. Designations: PYMV-CLT, Calicut isolate of PYMV; PYMV-WYD, Wynad isolate of PYMV; PYMV-IDK, Idukki isolate of PYMV; PYMV-KDG, Kodagu isolate of PYMV.**

infected *P. betle* and *P. longum* using DNeasy Plant mini kit (Qiagen) was used as template in PCR. PCR components were as explained earlier. Samples were amplified using a thermocycler (Eppendorf Master Cycler Gradient) using a reaction profile of

an initial denaturation at 94º C (3 min), followed by 35 cycles of denaturation at 94ºC (30 s), primer annealing at 58ºC/ 56º C (ORF I / ORF III) (1 min), primer extension at 72º C (1 min) and a final extension at 72º C (10 min). The amplified products were analyzed on 1% agarose gel containing ethidium bromide. A 450 bp and 600 bp products were seen corresponding to ORF-I and ORF-III respectively in both the cases (Fig. 14). The amplified fragments were eluted from the gel using Perfect prep gel extraction kit (Eppendorf). The purified product was then cloned into TA cloning vector (TA cloning kit, Genei, Bangalore) and selected recombinant clones were sequenced at Avestha GenGraine Technologies Pvt Ltd, Bangalore.

The sequenced region of ORF I in both the isolates contained 469 nucleotides. This region was compared with the PYMV isolates infecting black pepper and other badnaviruses. Pair wise comparison of ORF I region of both the isolates of *Badnavirus* infecting *P. betle* and *P. longum* revealed an identity of 87 % at the nucleotide level. ORF I region of PYMV black pepper isolate when compared with *P. betle* showed an identity of 99 %, while with *P. longum*, identity was 87%. The identity of both the isolates when compared with other badnaviruses was less than 40%.

The sequenced region of ORF III of *P. betle* and *P. longum* contained 597 nucleotides potentially coding for 199 amino acids. Pair wise comparison revealed an identity of 89 % between *P. betle* and *P. longum* isolates. Black pepper isolates of PYMV shared an identity of 94–97 % with *P. betle* and 88–89 % with *P. longum* at the nucleotide level. Percent identity of less than 60 % was seen with other badnaviruses used for comparison. Pair wise comparison of ORF III at the amino acid level revealed an identity of 93 % between *P. betle* and *P. longum*. PYMV isolate of *P. betle* shared an identity of 96-98% with black pepper isolates and PYMV isolate of *P. longum* showed comparatively low identity (93- 94%) with black pepper isolates. Less than 70% identity was seen with other badnaviruses. The results of multiple sequence alignments based on

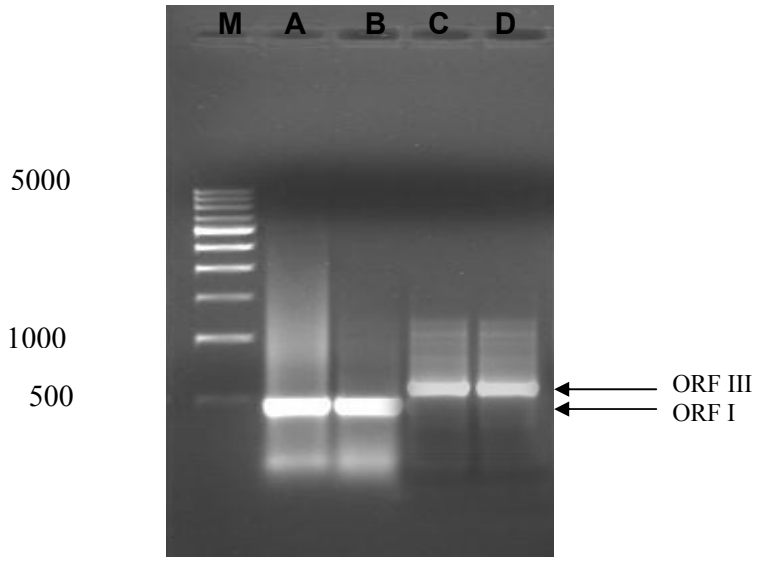


**Fig. 14. Agarose gel showing PCR products of ORF I and ORF III of *Badnavirus* infecting betel vine and Indian long pepper. Lane M: 500 bp DNA size ladder, Lane A: *P. betle* / ORF I, Lane B: *P. longum* / ORF I: - Lane C: *P.betle* / ORF III and LaneD: *P. betle* / ORF III.**

nucleotide sequence of ORF I and amino acid sequence of ORF III were used to generate phylograms. In both dendrograms, all isolates of *Piper* spp- *P. nigrum, P. betle, and P. longum* were clustered in a single group (Fig. 15, 16). However among the isolates, *P. betle* isolate was closer to black pepper isolates than to *P. longum* isolate. Thus based on both nucleotide and deduced amino acid sequences of portion of ORF- I and ORF-III, it was concluded that *Badnavirus* infecting *P. betle* and *P. longum* are strains of PYMV.

**9.0. Detection of CMV in black pepper**

**9.1 Standardization of protocol for isolation of total RNA from black pepper**

A reliable protocol for the isolation of total RNAs from black pepper was standardized for the use in RT-PCR based on acid guanidinum thiocynate– phenol – chloroform (AGPC) method with some modifications. 50mg of infected leaf was initially homogenized in 500µl denaturing solution (4 M guanidine thiocyanate, 25mM sodium citrate pH 7, 1% sarcocine, 0.5% β-mercapto ethanol) and subsequently, 50µl of 2 M sodium acetate (pH 4.0), 500µl of water saturated phenol and 100µl chloroform: isoamyl alcohol mixture (49:1) were added to the homogenate (with thorough mixing by inversion after addition of each reagent) and the final suspension was shaken vigorously for 10 seconds and incubated in ice for 15 min. After centrifugation at 10,000 g for 20 minutes at 4 °C, the aqueous phase was transferred to a fresh tube, mixed with 1 ml of isopropanol, and incubated at –80 °C for 30 minutes to precipitate the RNA. The RNA pellet obtained after centrifugation at 10,000 g for 20 minutes at 4 °C was dissolved in denaturing solution and again the above steps were repeated with sodium acetate, water saturated phenol and chloroform: isoamyl alcohol and precipitation with isopropanol. The pellet was washed with 75% ethanol, dried and dissolved in 50µl of RNase free water.

**9.2 Standardization of RT-PCR for detection**

The method was standardized wherein RT and PCR was performed in the same tube without any buffer changes in between. The primer pair designed from the CP gene sequence of CMV (black pepper isolate) was used in the reaction to prime the amplification. The genome sense primer 5′ATGGACAAATCTGAATCAAC 3′ derived from the beginning of first 20 bases of the coding region and the genome antisense primer 5′ TCAAACTGGGAGCACCC 3′ represents the last 17 bases of the CP gene. The PCR reaction (50 µl) contained 30 pmole each of the primers, 10 units Ribonuclease inhibitor, 5 units AMV reverse transcriptase, 1.5 units *Taq* Polymerase, 1x PCR buffer, 10 mM Dithiothreitol, and 10 µM each of the dNTPs. PCR mix (13.5 µl) containing the above components was added to the tubes containing the template RNA (36.5µl) resulting in a final reaction volume of 50 µl. Amplification was performed in an automated thermal cycler and the programme consisted of one cycle at 42 °C for 45 min for cDNA synthesis followed by 40 cycle reaction profile involving 30 s of denaturation at 94 °C, 1 min of annealing at 50 °C, and 1 min of extension at 72 °C and a single cycle of final extension at 72 °C for 10 min. Results of RT-PCR showed successful amplification of CMV coat protein gene and a PCR product of expected size (650 bp) was observed only in the infected black pepper samples.

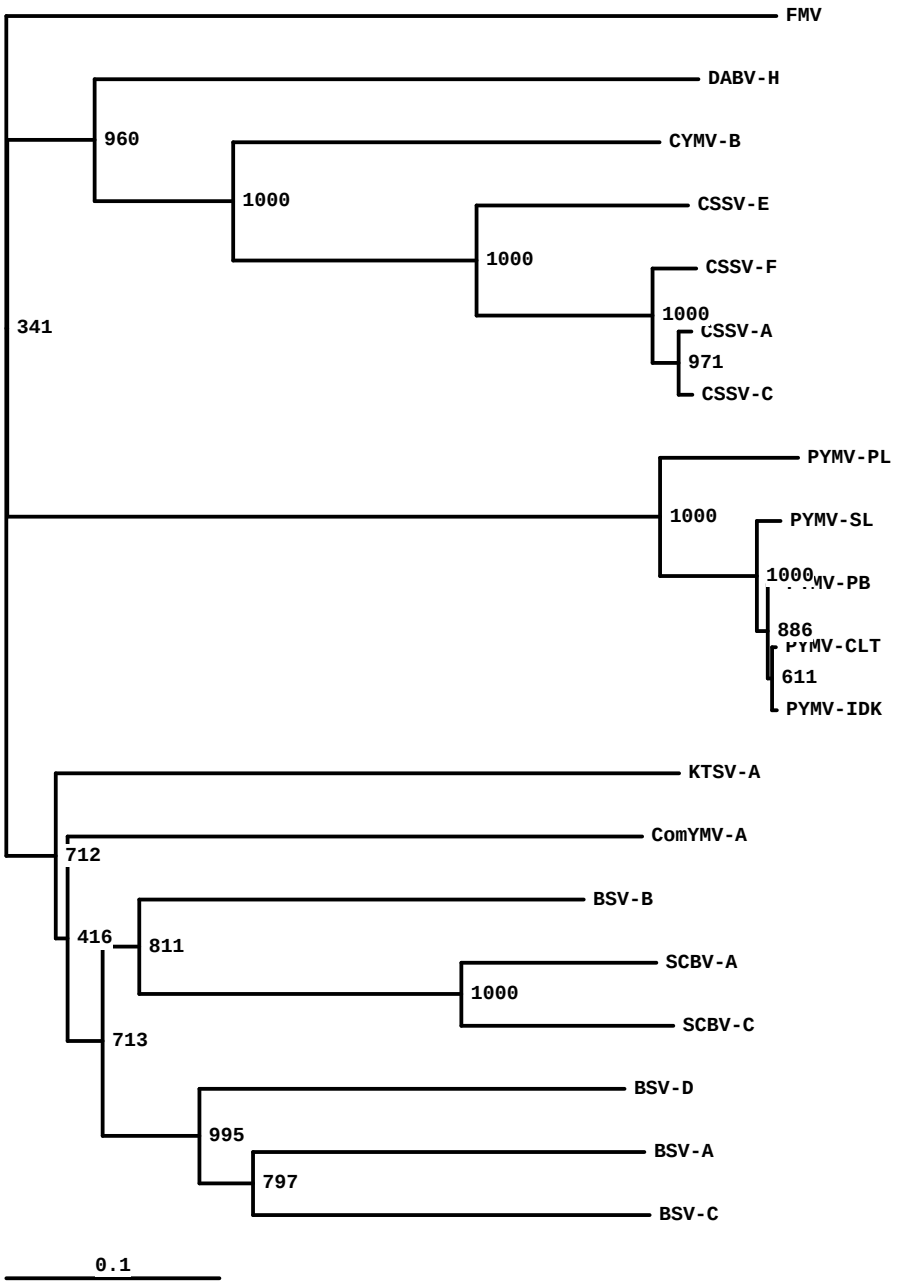


**Fig. 15. Phylogram drawn by Neighborhood Joining Bootstrap method in Clustal X (1.81), showing phylogenetic relationships based on multiple alignments of ORF I nucleotide sequences of *Badnavirus* infecting *Piper betle* (PYMV-PB) and *P. longum* (PYMV-PL) with different badnavirus species and their strains. FMV was used as outgroup. The bootstrap values are shown at the individual nodes.**

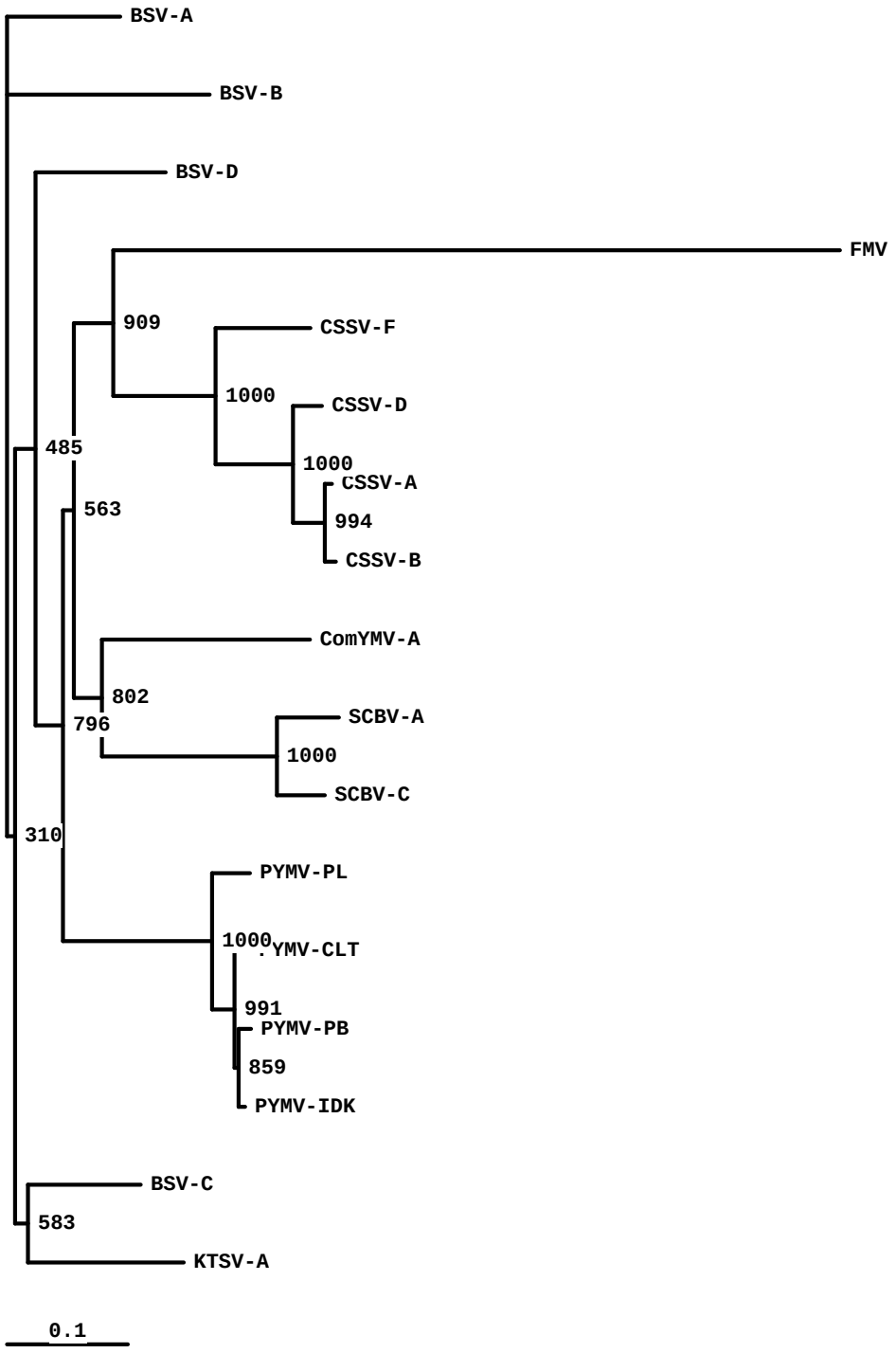


**Fig. 16. Phylogenetic tree depicting relationship of *Badnavirus* infecting *Piper betle* (PYMV-PB) and *P. longum* (PYMV-PL) with various badnaviruses and their strains based on multiple sequence alignment of ORF III amino acid sequences. FMV was used as outgroup. The bootstrap values are shown at the individual nodes**

### 9.3. Determination of optimum template RNA concentration for RT-PCR

In order to determine the optimum template RNA required for successful amplification, RT-PCR was performed with different quantities of templates such as 0.01, 0.05, 0.1, 0.5, 1, 5, 10 and 36.5µl. RT-PCR could successfully detect CMV in all quantities of templates used except in 0.01µl (Fig. 17). Increased template quantity from 0.05 to 1µl resulted in higher intensity of the expected band while further increase in the template quantity did not result in proportional increase of the expected PCR band. Thus use of template RNA at 1µl was found to be optimum for the detection of CMV. When quantified, total RNA concentration in different preparations varied from 790 to 920 ng/µl

### 9.4. Detection of CMV in different black pepper plants and plant parts

In order to know the distribution of the CMV within different plant parts, RT-PCR was performed with template RNA extracted from young leaf, old leaf, stem and root. Results showed the presence of CMV in all plant parts tested thus confirming systemic nature of the virus. Further, in order to test the utility of RT-PCR protocol in the detection of CMV in field samples, RT-PCR was carried out using template RNA extracted from different field samples. Results of this clearly showed that the method standardized could be successfully utilized for detection of CMV in field samples (Fig. 18)

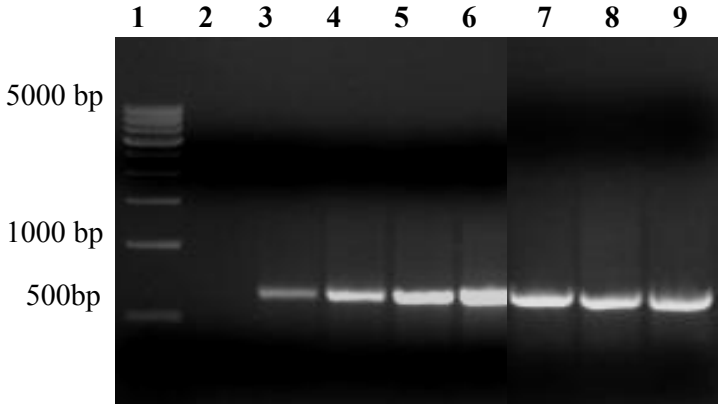


**Fig. 17. Detection of CMV with varying concentration of RNA template by RT-PCR. Lane 1: 500 bp DNA ladder, Lane 2-9: RT-PCR with varying template quantities such as 0.01µl, 0.05µl, 0.1µl, 0.5µl, 1µl, 5µl, 10µl, and 36.5µl respectively.**

## 10.0. Detection of PYMV in black pepper

### 10.1. Standardization of total DNA isolation protocol from black pepper

A reliable protocol for the extraction of total DNA from black pepper was standardized by modifications of the protocol described by de Silva et al. (2002). In the modified protocol 100 mg of leaf tissue from infected plants were ground in 500µl of CTAB buffer (100mM Tris HCl pH 8, 4mM EDTA pH 8, 1.4 M NaCl, 2% CTAB, 1% PVP, 0.5% β-mercaptoethanol) and incubated at 65 °C for 30 min. The homogenate was later allowed to cool to room temperature and extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and centrifuged at 2500g, for 10 min at room temperature and the supernatant was collected and 0.1

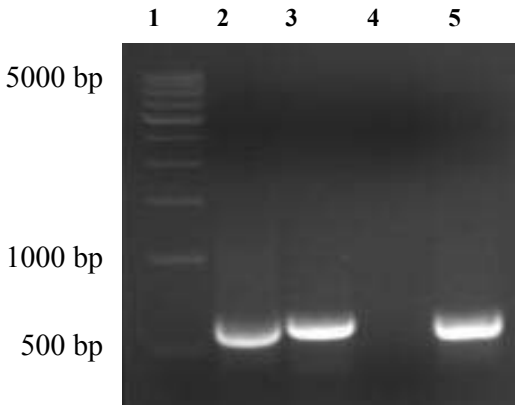


**Fig. 18. Detection of CMV in black pepper plants. Lane 1: 500 bp DNA ladder, Lane 2 -5: RT-PCR product obtained from different black pepper plants from field.**

volume of 10% CTAB was added. This mixture was later re-extracted using chloroform: isoamyl alcohol (24:1) followed by centrifugation at 2500g for 10 min at room temperature. The supernatant was collected and to this 0.1 volume of 3 M sodium acetate (pH 5.2) and an equal volume of 100% isopropanol were added and incubated in ice for 30 min for the DNA to precipitate. The DNA was palletized by centrifuging at 10,000 rpm for 15 min at 4 °C and the obtained pellet was washed in 70% ethanol, air dried and dissolved in 100µl sterile distilled water.

### 10.2 Standardization of PCR for detection

The primer pair F 5′ CTCCTTCATCTCCTCAAGAAGCCT 3′ and R 5′ CCAAAGCTCTGATAGCAGAC 3′ for PCR detection of the badnavirus was designed based on the ORF I sequence of badnavirus infecting black pepper in India. The PCR reaction contained 1x PCR buffer, 2.5mM $MgCl_2$, 10 µM each of dNTPs, 50 ng each of forward and reverse primers, 1.5 Units of Taq DNA polymerase and 5µl of template and sterile water to a final volume 50µl. The thermal cycler was programmed for initial denaturation at 94 °C for 5 min, followed by 34 cycles of denaturation at 94 °C for 30 sec, annealing at 56 °C for 1 min, synthesis at 72 °C for 1 min and a final extension for 10 min at 72 °C. The PCR was successful in detecting the virus as band of ~700 was seen only in infected black pepper.

### 10.3. Determination of optimum concentration of template DNA for PCR

To estimate the optimum quantity of the template needed to be used in the PCR for detection, DNA was extracted from 100 mg of leaf tissue, finally dissolved in 100 µl of sterile distilled water and used in PCR at different quantities ranging from 0.01µl to 30µl. The different template concentrations tried were 0.01, 0.05, 0.1, 0.5, 1, 2, 3, 4, 5, 10, 15, 20, 25, and 30µl. The PCR gave amplified products of expected size (700 bp) in all the template concentrations except in 30µl. (Fig. 19). The quantity of template required for the better

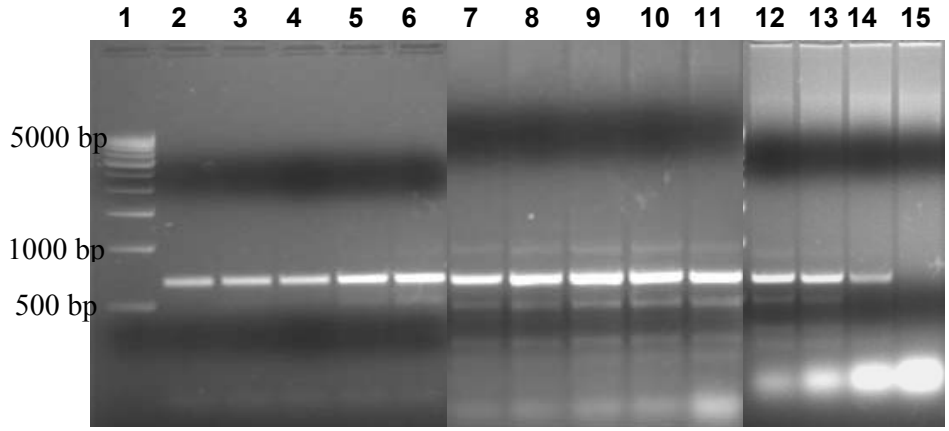


**Fig. 19. Detection of PYMV in black pepper using varying concentration of the template DNA. Lane 1: 500 bp marker, lane 2-15: PCR product with varying template DNA quantities such as 0.01µl, 0.05µl, 0.1µl, 0.5µl, 1µl, 2µl, 3µl, 4µl, 10µl, 15µl, 20µl, 25µl, 30µl.**

amplification of PYMV was found to be in the range 0.5µl to 15µl. When quantified spectrophotometrically, the DNA concentration in the above preparation was found to be 130 ng/µl. The study suggested the possibility of using a wide range of template concentration from 65 to 1950 ng. (i.e 0.5 to 15 µl of the template) for better and consistent detection of the virus in plants.

### 10.4. Detection of PYMV in field samples of black pepper.

In order to examine the reliability of PCR for screening plants for the presence PYMV in nursery planting material and field plants, DNA was isolated from various test plants using the protocol described above and PCR was performed using 5 µl template DNA, along with a known healthy (negative control) and infected (positive control) samples. Results showed PCR protocol standardized was efficient in detecting the virus in various plants (Fig. 20).

## 11.0. Development of multiplex RT-PCR for simultaneous detection of CMV and PYMV infecting black pepper.

A reliable protocol for the simultaneous isolation of total nucleic acids (DNA & RNA) from black pepper in a single tube was standardized. A multiplex RT-PCR based detection methodology was developed for the detection both the viruses, CMV and PYMV from black pepper. Primer pairs specific for coat protein gene of CMV and a portion of ORF I of PYMV were used to prime the reaction. This standardized multiplex RT-PCR reaction gave two bands of ~ 650 bp (corresponding to CMV) and ~450 bp (corresponding to PYMV) (Fig. 21).

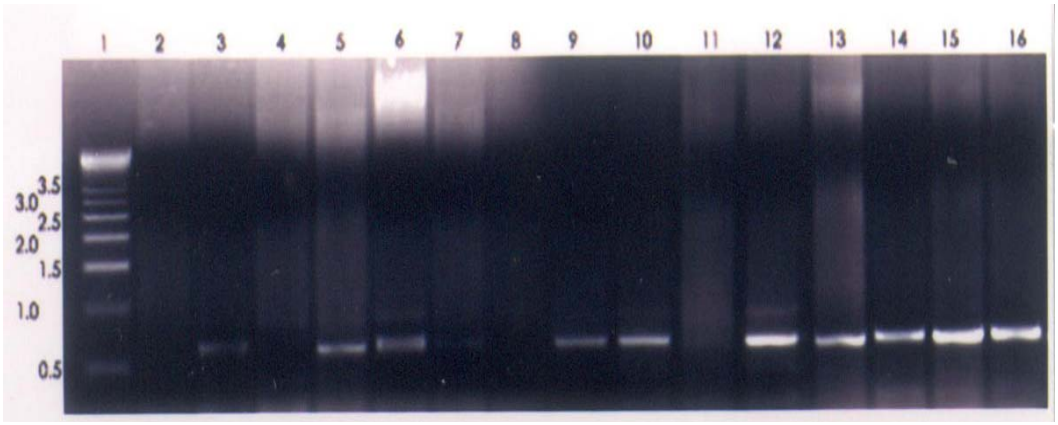


**Fig. 20. Detection of *Piper yellow mottle virus* in different plants. Lane 1. 500bp marker, lane: 2 negative control (healthy black pepper), lanes 3-15: test plants, lane 16: positive control (PYMV infected black pepper).**

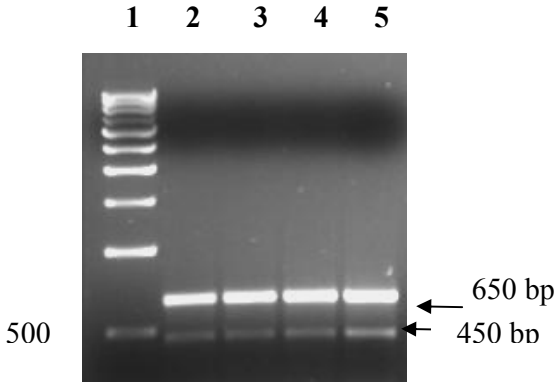


**Fig 21. Detection of CMV and PYMV from infected black pepper samples using multiplex RT-PCR. Lane 1: 500 bp ladder, Lane 2-5: Infected black pepper plants .The 650 bp is specific to CMV and 450bp band is specific to PYMV.**

### 11.1. Determination of optimum concentration of the template for multiplex RT-PCR

Optimum template volume for successful multiplex RT-PCR ranged from 0.1µl to 1µl and optimum template concentration was estimated to be ranging from 0.1µg to ~1µg in a reaction volume of 25µl (Fig 22).

### 11.2 Detection of CMV and PYMV in field samples of black pepper

In order to examine the reliability of multiplex RT-PCR for screening plants for the presence CMV and PYMV in nursery and field plants, the total nucleic acid was isolated from various test plants and multiplex RT-PCR was performed along with a known healthy (negative control) and infected (positive control) samples. Results showed PCR protocol standardized was efficient in detecting the viruses in various plants (Fig. 23). Thus this single tube m-RT-PCR can be used in indexing

black pepper to identify virus free plants. The technique is highly sensitive and is economical in the use of reagents and hence may be developed as a diagnostic kit for detection of viruses in black pepper.

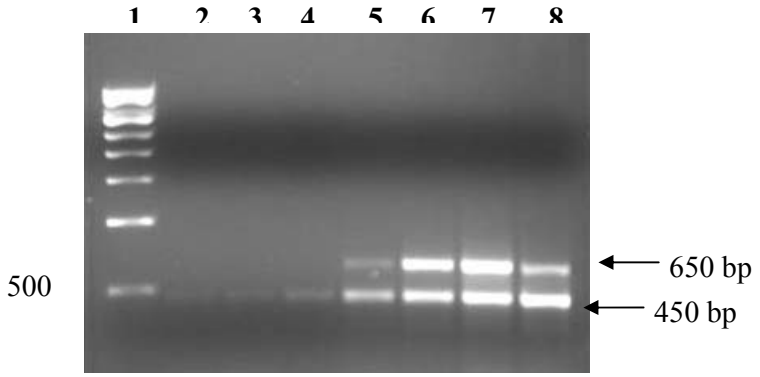


**Fig.22. Optimization of template concentration for multiplex RT-PCR. Lane 1: 500 bp marker, Lane 2- 8 different template concentration: 10pg, 100 pg, 1ng, 10ng, 100ng 1µg, 10µg.**

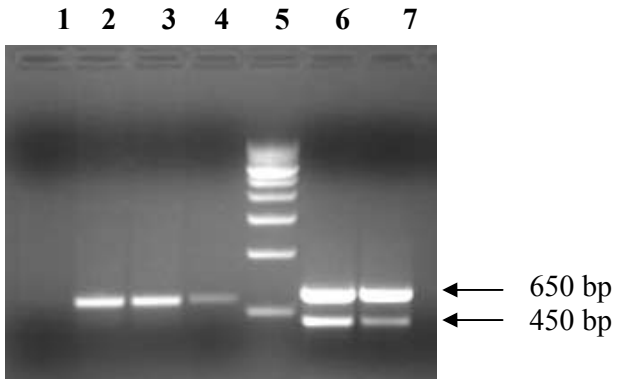


**Fig. 23. Screening of field plants using multiplex RT-PCR. Lane 1: Healthy black pepper (negative control), Lane 5: 500 bp marker, Lanes 2, 3, 4 and 6: various test plants, Lane 7: Infected black pepper (positive control).**

## 8222 Questions- Answered

The stunted disease is caused by two viruses namely, *Cucumber mosaic virus* and *Piper yellow mottle virus*

CMV sequences were highly conserved among various isolates of the virus from different *Piper* spp. While in PYMV, sequences were conserved in ORF-III while it variable in ORF-I.

A reliable detection methodology developed for detecting both the viruses in a single PCR reaction.

**8223 Process/ Product/ Technology/ Developed**

A reliable protocol was developed for the isolation of total RNAs from the black pepper plants and RT-PCR procedure for efficient detection of CMV

The protocol for the extraction of total DNAs and PCR was developed for detection of PYMV in infected black pepper plants.

An efficient protocol for total nucleic acid isolation and a multiplex PCR based detection methodology was developed for the simultaneous detection of CMV and PYMV causing stunted disease in black pepper.

**8224 Practical Utility (not more than 150 words)**

For management of stunted and phyllody disease use of virus-free planting materials is important. As symptoms can not be reliably used to identify healthy plants, use of sensitive methods based on PCR is needed to identify virus-free plants. The project developed a reliable and sensitive multiplex reverse transcription polymerase chain reaction methodology for the detection of both the viruses which can be used in certification programme to develop virus-free planting materials.

**Constraints, if any**: Nil

**823 Publications and Material Development**

(One copy each to be supplied with this proforma.)

8231 Research papers

Bhat, A.I., Hareesh, P.S. and Madhubala, R. 2005. Sequencing of coat protein gene of an isolate of *Cucumber mosaic virus* infecting black pepper (*Piper nigrum* L.) in India. *Journal of Plant Biochemistry and Biotechnology*, **14:** 37-40.

Hareesh, P.S., Madhubala, R. and Bhat, A. I. 2006. Characterization of *Cucumber mosaic virus* infecting Indian long pepper (*Piper longum* L.) and Betel vine (*Piper betle* L.). *Indian Journal of Biotechnology*, **5:** 89-93.

Siju, S., Madhubala, R., Bhat, A.I. Sodium sulphite enhances nucleic acid isolation and sensitivity of virus detection by RT-PCR in black pepper. J Virol Methods (in press)

**Manuscripts communicated**

Hareesh. P.S. and Bhat, A.I. Molecular characterization, variability and PCR detection of the *Badnavirus* infecting black pepper (*Piper nigrum* L) in India.

Siju, S., Bhat, A.I. and Hareesh, P.S. Detection and characterization of *Badnavirus* infecting betel vine (*Piper betle* L.) and Indian long pepper (*P. longum* L.) in India.

**8232 Popular articles**

Bhat, A.I. 2005. Polymerase chain reaction and its application for plant virus detection. Spice India 18 (2):47-49.

Bhat , A.I., Devasahayam, S. and Anandaraj, M., 2003. Viral disease- a new threat to black pepper cultivation in India. *Indian J. Arecanut, spices and Medicinal plants*. 5(2): 46-48.

Bhat, A.I. 2003. Diagnostics for virus detection-its importance in spices. *Spice India.* 16 (9): 30-33.

8233 Reports
Nil

8234 Seminars, conferences and workshops (relevant to the project) in which the scientists have participated. (List abstracts forwarded)
Nil

**824 Infrastructural facilities developed**
(Details of field, laboratory, note books and final material and their location)
Four instruments namely, vacuum concentrator, -20 freezer, shaker incubator and a microcentrifuge were purchased under this project

825 Comments / Suggestions of Project Leader regarding possible future line of work that may be taken up arising out of this Project.

As badnaviruses are known to integrate into their host genome, it is important to study whether such a phenomena occur in PYMV-black pepper also. The PCR method developed for detection of the viruses should be used in certification programme to identify virus-free planting material. Identification of resistant sources against the viruses in the *Piper* germplasm and their use in breeding to get virus resistant lines should be carried out. As an alternative to this, pathogen mediated transgenic approach need to be worked out to get virus resistant black pepper cultivars.

**Part-IV : Project Expenditure** (2003-06)
(Summary)

| **Head** | **Grant allotted** | **Total grant released till date** | **Grant utilized** | **Balance** |
|---|---|---|---|---|
| Non-recurring | 5,20,345 | 5,20,345 | 5,20,345 | 0 |
| Manpower | 6,73,000 | 6,52,000 | 6,19,300 | 32,700 |
| Recurring contingencies | 1,20,000 | 1,20,000 | 1,20,000 | 0 |
| Consumables | 9,00,000 | 7,70,000 | 7,99,522 | -29,522 |
| TA | 60,000 | 40,000 | 40,000 | 0 |
| Institutional charges | 2,00,000 | 2,00,000 | 2,00,000 | 0 |
| **Total** | **24,73,345** | **23,02,345** | **22,99,167** | **3,178** |
| Balance refunded | | | | **3,178** |

**Part-V : Declaration**

This is to certify that the final report of the Project has been submitted in full consultation with the Project workers as per the approved objectives and technical programme and the relevant records, note-books, materials are available for the same.

Signature of the Project Investigator: A. Ishwara Bhat

Co-Investigator: R. Suseela Bhai

Signature & Comments of the Head
of the Division/ Section

Signature & Comments of the
Director