FINAL REPORT OF RESEARCH PROJECT

Development of diagnostics for viruses infecting small cardamom (*Elettaria cardamomum* Maton)

PRINCIPAL INVESTIGATOR

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RPF - III

PROFORMA FOR SUBMISSION OF RESEARCH PROJECT (S)

Part-1: General Information

800. Project code : Path XIX (813)

8001. Institute Code No. : Path XIX (813)

8002. ICAR Code No :

801. Name of Institute and Division:

8011. Name & Address of Institute: Indian Institute of Spices Research, Kozhikode, 673 012

8012. Name of Division/Section: Division of Crop Protection

8013. Location of Project: IISR, Kozhikode, Kerala and Cardamom Research Centre, Appangala, Karnataka

802. Project Title: Development of diagnostics for viruses infecting small cardamom (*Elettaria cardamomum* Maton)

803. Priority Area: Development of diagnostic tools

Applied Research	Basic Research	Process/Technology Development	Transfer of Technology
01*	02*	03 *	04

8031 Research/Approach : 01, 02, 03

804. Specific area: Cardamom plant protection

805. Duration : 4 years

8051. Date of start : 01-04- 2008

8052. Date of completion : 31-03-2012

806. Total cost/ Expenditure incurred: ₹ 14, 07,000

(Give reasons for variation, if any from estimated cost)

Due to the enhancement of pay of the scientific personnel involved.

807. Executive summary:

Small cardamom (*Elettaria cardamomum* Maton) is affected by a number of fungal, bacterial and viral diseases, which inflict damages to a considerable extent to the crop. Among the viral diseases, crop losses caused by katte/ mosaic disease incited by *Cardamom mosaic virus* (CdMV) is a major production constraint for cardamom in India

and elsewhere. Besides katte, chlorotic streak disease incited by a strain of *Banana bract mosaic virus* (BBrMV) is an emerging disease which could be a possible threat to the industry in future. Cardamom is propagated mainly by vegetative means and the viruses infecting cardamom primarily spreads through the planting material. Once infected, the viruses become systemic in the plant and the infected planting material serves as primary source of inoculum in the field. Once the primary foci of infection are established in the field, viruliferous vectors mediate secondary spread of the viruses to healthy plants. Hence, in order to obtain virus-free planting material, it is highly essential to index the mother stock with appropriate and sensitive techniques. Thus, identification and characterization of viruses associated with the diseases and development as well as standardization of diagnostic tools for each identified virus is of paramount importance. Once developed and standardized, these tools can be subsequently used for indexing the planting material and identifying resistant lines/ cultivars before they are further multiplied in large scale or incorporated in the breeding programmes.

During the surveys undertaken in major cardamom cultivating tracts of Karnataka, Kerala and Tamil Nadu of South India, incidence of mosaic disease was found in the range of 0-85%. Twenty four CdMV isolates and five BBrMV isolates were collected and subsequently planted in the insect proof glass house condition for characterization studies.

Studies on symptoms and sequence analysis of coat protein (CP) gene of CdMV isolates from different geographical locations revealed the existence of high diversity among the isolates. Analysis of the isolates based on symptoms and CP gene sequence indicated region wise variation among the isolates.

Protocol for purifying CdMV was standardized. Polyclonal antiserum was produced against CdMV and ELISA based detection of CdMV was standardized. Protocols for RNA isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for detecting CdMV in the mosaic disease affected cardamom plants were standardized and validated by testing 53 field samples originating from different geographical locations

Surveys undertaken in the major cardamom cultivating tracts of Karnataka, Kerala and Tamil Nadu of South India revealed that the incidence of chlorotic streak disease was in the range of 0-15%.

Symptomatological studies indicated that, the symptoms induced by BBrMV on cardamom were distinct characterized by the appearance of chlorotic streaks along the veins. Association of BBrMV with symptomatic cardamom plants was revealed through electron microscopy. Coat protein gene sequences from five different isolates of the virus showed > 94% identity with BBrMV clearly indicating that causal virus is a strain of BBrMV. Sequence identity and phylogenetic relationship studies showed close relationships among isolates irrespective of their geographic origin or host indicating that the virus had a single common origin. Protocols for RNA isolation and RT-PCR for detecting BBrMV in the cardamom plants were standardized and validated by testing samples originating from field.

Transmission studies of BBrMV infecting cardamom using aphids as well as by sap inoculation indicated non-transmission of the virus either through sap or by the aphids, which was subsequently confirmed by real time-PCR. Seed transmission studies of CdMV and BBrMV were carried out using immature capsules, immature and mature seeds collected from virus infected plants of cv. Njallani Green Gold. Though presence of both the viruses could be detected in the seed parts when tested with RT-PCR, the seedlings raised from them were asymptomatic and the absence of both the viruses in the seedlings was confirmed by performing RT-PCR, indicating non- transmission of both BBrMV and CdMV through seeds. Real time-PCR based procedure for the detection of CdMV and BBrMV was standardized. Multiplex RT-PCR based procedure for the simultaneous detection of CdMV and BBrMV was also standardized

808. Key Words: Katte/mosaic disease, chlorotic streak, *Cardamom mosaic virus*, *Banana bract mosaic virus*, Reverse Transcription-Polymerase Chain Reaction, real time PCR, diagnostics, seed transmission, aphid transmission, sap transmission, multiplex PCR.

PART-II: INVESTIGATOR PROFILE

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

810. Principal Investigator

8101. Name: C.N. Biju
8102. Designation: Scientist (Plant Pathology)
8103. Division/Section: Crop Protection/Plant Pathology
8104. Location: IISR, Cardamom Research Centre, Appangala, Karnataka
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811. Co- Investigator

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8112. Designation: Senior Scientist (Plant Pathology)
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8115. Institute Address: Indian Institute of Spices Research, Kozhikode, Kerala, 673 012

PART- III: TECHNICAL DETAILS

820. Introduction and Objectives

8201. Project objectives:

(i) To develop immuno based diagnostics for the viruses infecting cardamom.

(ii) To develop nucleic acid based diagnostics for the viruses infecting cardamom.

(iii)To develop multiplex-PCR for simultaneous detection of viruses infecting cardamom.

8202: Background information and importance of the project:

Small cardamom (*Elettaria cardamomum* Maton) suffers from the attack of several fungal, bacterial and viral diseases, which results in considerable crop losses. Among the viral diseases, crop losses caused by katte/ mosaic disease, incited by *Cardamom mosaic virus* (CdMV) is a major production constraint for cardamom in India and elsewhere. Besides katte disease, chlorotic streak caused by *Banana bract mosaic virus* (BBrMV) is an emerging disease which might pose threat a to the cardamom industry in future. Cardamom is propagated mainly by vegetative means and the viruses infecting cardamom primarily spreads through the planting material. Moreover, identification and characterization of viruses associated with the diseases is also of

paramount importance for developing reliable and sensitive diagnostic tools for the fool-proof detection of the viruses associated with respective diseases. Development of sensitive diagnostic tools for detection of viruses in the planting material is highly essential in order to index the mother stock and ascertain its virus-free status. Hence, the project was formulated with major objectives to develop diagnostic tools for the viruses infecting cardamom.

821. Project Technical Profile:

8211. Technical programme:

(Indicate briefly plan of procedure, techniques, instruments and special materials, organisms, special environment etc.)

1. Collection, establishment and maintenance of CdMV and BBrMV isolates in insect proof glass house conditions.
2. Purification of CdMV particles and production of polyclonal antiserum against CdMV.
3. Development of Enzyme Linked Immunosorbent Assay (ELISA) based protocol for the detection of CdMV.
4. Development of Reverse Transcription- Polymerase Chain Reaction (RT-PCR) based detection of CdMV.
5. Validation of RT-PCR based protocol for the detection of CdMV by testing field samples of cardamom.
6. Development of nucleic acid based detection of BBrMV infecting cardamom.
7. Transmission studies of BBrMV by aphids and through sap inoculation.
8. Seed transmission studies of CdMV and BBrMV in cardamom.
9. Development of Real-time PCR based detection of BBrMV and CdMV.
10. Development of Multiplex RT-PCR (mRT-PCR) protocol for the simultaneous detection of CdMV and BBrMV.

8212. Man month involvement of component project workers for the specified year:

(a) Scientific : 28

(b) Technical : 08

(c) Supporting : 08

822. Progress of work:

8221. Achievement in terms of targets fixed for each activity:

Katte/ marble/ mosaic disease (*Cardamom mosaic virus*)

(I) Survey, incidence and symptomatology

Survey and incidence of mosaic disease

A total of 84 cardamom plantations belonging to 44 geographical locations of Karnataka and Kerala were surveyed to study distribution and incidence of katte/ mosaic disease.

For collection of the samples from each locality, the plantations were randomly selected to represent different cardamom growing locations. In each selected plantations, disease incidence was recorded based on the observations on a cluster of 100 plants. The number of clusters in each plantation was fixed on the basis of the effective area under cardamom cultivation; 2 ha = 5 clusters; 2.1 to 5 ha = 10 clusters; 5.1 to 10 ha = 15 clusters and more than 10 ha = 20 clusters. The disease incidence in each plantation was calculated after the counts of all the clusters were pooled. Care was taken to ensure even distribution of sampling units to represent different parts of plantations (Govindaraju *et al.*, 1994).

In general, the incidence of viral disease was higher in Karnataka compared to Kerala (Table 1). Of the 84 plantations in 44 locations surveyed, incidence of the disease ranged from 0-85%. In Karnataka, of the 25 fields belonging to 13 locations surveyed, disease incidence ranged from 2 to 85%. In Kodagu district, the lowest incidence of 2% was recorded at Horur which belongs to Somwarpet taluk and the highest incidence of 85% was recorded at Bhagamandala of Madikeri taluk. In Hassan district, out of 12 plantations surveyed, katte was found to be wide spread with an incidence ranging from 30 to 73%.

In Kerala, surveys were conducted in two major cardamom growing districts *viz.*, Wayanad and Idukki. Out of 59 fields belonging to 31 locations surveyed revealed that, the incidence of katte was less and spread was restricted. In Idukki district, the disease was mainly confined to Mankulam, Vathikudy, Kattappanan, Pampadumpara and Myladumpara villages of Udumpanchola, Kattappana and Devikulam taluks with disease incidence ranging between 2 to 12 %. In Wayanad district, out of 11 fields belonging to 5

locations surveyed, disease was observed only in Muppayinad village with 2% disease incidence. During the survey, representative isolates of the virus i.e., 24 isolates (17 from Karnataka and 7 from Kerala) were collected and established under insect proof glass house conditions (Table 2).

Table 1: Distribution and incidence of katte disease on small cardamom in Karnataka and Kerala

State/ District	Taluk	Locations	No. of fields surveyed	Disease incidence	
				Range (%)	Mean (%)
Karnataka					
Hassan	Sakleshpur	8	12	30–73 %	48.58
Kodagu	Somwarpet	2	3	2 – 15 %	10.00
	Virajpet	1	2	17 – 26 %	21.50
	Madikeri	2	8	32 – 85 %	62.00
Kerala					
Idukki	Udumpanchola	17	37	0 – 6 %	0.54
	Peermade	4	4	0 %	0.00
	Devikulam	5	7	0 – 12 %	1.71
Wayanad	Sulthan Barhery	4	8	0 %	0.00
	Vythiri	1	3	0 – 2 %	0.66

Table 2: Places of collection of *Cardamom mosaic virus* (CdMV) isolates

State	Locations
Karnataka	Jodpal
	Kakkabe
	Heravanadu (Appangala)
	Murnad
	Hebbettageri
	Kundally
	Nagarahally
	Ballehalla
	Kyanahally
	Hullahally
	Kunigal
	Bobbanahally
	Horur
	Bhagamandala (4 isoalates)
Kerala	Kattappana
	Myladumpara (3 isolates)
	Suryanelli (Chinnakanal)
	Mankulam
	Wayanad

Symptomatology of mosaic disease

In Karnataka, SKP-14 (ICRI – 3) and CCS – 1 (IISR Kodagu Suvasini) were the varieties grown on large scale, while the cultivar Njallani Green Gold was confined to certain locations while, in Kerala, Njallani Green Gold was the widely adopted and grown cultivar. The symptoms induced by CdMV on ICRI - 3 and IISR Kodagu Suvasini were similar and the symptoms included prominent light green/ yellow mosaic patches along the veins. However, there were variations in the symptoms induced by CdMV on Njallani Green Gold grown in Karnataka and Kerala. In Karnataka, the symptoms included light green with prominent chlorotic streaks (Kodagu) (Plate 1) while, prominent light green/yellow mosaic patches along the veins (Idukki) were the symptoms induced by CdMV on Njallani Green Gold cultivated in Kerala (Plate 2). The other major symptoms like mottling of leaf sheath and pseudostem and shorter and slender tillers were found to be common in all the plantations surveyed in Karnataka and Kerala, irrespective of the cultivars or varieties grown. Six isolates viz., Sirsi, Sakleshpur, Appangala, Meppadi, Pampadumpara and Thadiyankudisai with distinct symptoms (Table 3) collected during the surveys were subsequently used for coat protein gene sequence studies.

Plate 1

Plate 2

Table 3: Symptoms observed on different *Cardamom mosaic virus* isolates used for coat protein gene sequence studies

Isolate	Region (District, State)	Variety/cultivar	Symptoms on	
			Leaves	Pseudostem/ leaf sheath
Sirsi	Uttara Kannada, Karnataka	ICRI – 3	Whitish green with discontinuous dark green islands	Mild mosaic mottling
Sakleshpur	Hassan, Karnataka	ICRI – 3	Prominent yellow mosaic patches along the veins	Mild mosaic mottling
Appangala	Kodagu, Karnataka	Njallani Green Gold	Light green with prominent chlorotic streaks	Severe mosaic mottling
Meppadi	Wayanad, Kerala	Njallani Green Gold	Light green with prominent chlorotic streaks	Severe mosaic mottling
Pampadumpara	Idukki, Kerala	Njallani Green Gold	Continuous dark green stripes along the veins	Mild mosaic mottling
Thadiyankudisai	Dindigul, Tamil Nadu	Njallani Green Gold	Prominent yellow mosaic patches along the veins	Mild mosaic mottling

(II) Characterization of different geographical isolates of CdMV by coat protein gene sequencing and seed transmission studies

Characterization of geographical isolates of CdMV by coat protein gene sequencing

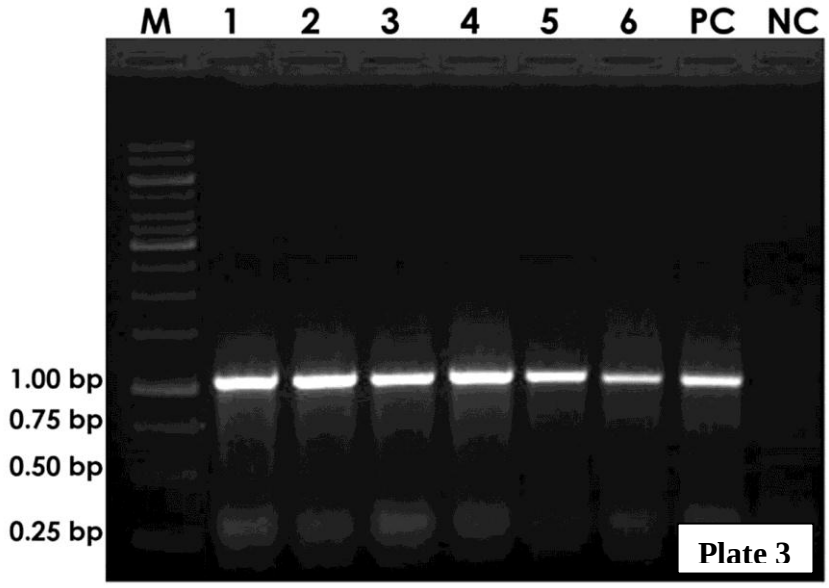
During the surveys conducted in major cardamom growing regions covering 84 plantations in 44 geographical locations of Karnataka, Kerala and Tamil Nadu of South India, six symptomatologically distinct isolates were collected from different regions. The regions included Sirsi (Uttara Kannada), Sakleshpur (Hassan), Appangala (Kodagu) of Karnataka, Meppadi (Wayanad), Pampadumpara (Idukki) of Kerala and Thadiyankudisai (Dindigul) of Tamil Nadu. These isolates were established under insect proof glass house conditions and were subsequently used for coat protein gene sequence diversity analysis.

Total RNA was isolated following the method described by Siju *et al.* (2007). To amplify coat protein gene of the CdMV, forward primer (5´CHCARCADTTTCARATGG 3´) that represented the last 18 bases of the NIb region and the reverse primer (5´CTCAGCAATTTCAATGG3´) derived from the 3´ untranslated region (UTR) were used. RT-PCR mixture contained 20 pmol of each of the forward and reverse primers, 10 U ribonuclease inhibitor (Fermentas, USA), 20 U M-MuLV reverse transcriptase (Fermentas, USA), 1.5 U *Taq* DNA polymerase (Fermentas, USA), 1 x PCR buffer (Genei, Bangalore), 10 mM dithiothreitol (Genei, Bangalore) and 25 µM each of the dNTPs (Genei, Bangalore). RT-PCR mix (40 µl) containing the above components was added to the tube containing template RNA (10 µl) resulting in a final reaction volume of 50 µl. Amplification was performed in an automated thermal cycler (Eppendorf master cycler gradient) and the program consisted of one cycle at 42 C for 45 min for cDNA synthesis followed by a 35 cycle reaction profile involving 30 s of denaturation at 94 C, 1 min of annealing at 56 C and 1 min of extension at 72 C and a single cycle of final extension at 72C for 10 min. The reaction products were analysed on 0.8% agarose gel along with 1Kb DNA ladder. The DNA bands were visualized and photographed using UV transilluminator and gel documentation apparatus (Alpha Innotech Corporation, USA).

The RT-PCR product was eluted from the gel using GenElute Gel Elution kit (Sigma-Aldrich, Bangalore), cloned into pTZ57R/T cloning vector (Fermentas, USA) and transformed into competent *E. coli* strain DH-5α using InsTAclone PCR cloning Kit (Fermentas, USA) following manufacturer's instructions. Recombinant clones were identified by PCR as well as restriction endonuclease digestion and selected clones were sequenced from both ends at the automated DNA sequencing facility available at Chromous Biotech, Bangalore. Multiple sequence alignments were made using ClustalX (1.81). Percent identities were determined using Bioedit program (version 5.0.9). Phylogenetic tree was constructed using Neighborhood algorithm in Clustal X with bootstrap analysis (1000 replicates). The coat protein nucleotide and amino acid sequences of other CdMV isolates used for comparison were obtained from GenBank

(Benson *et al.* 1999). The BLAST programme (Altschul *et al*. 1997) was used to identify related sequences available from the GenBank database.

RT-PCR amplified an amplicon of ~1050 bp in all the six isolates while no product was found in healthy (negative) control (Plate 3).



Lane M: Marker (1 kb ladder); Lanes 1-6: Infected isolates from Sirsi, Sakhleshpur, Appangala, Meppadi, Pampadumpara and Thadiyankudisai respectively; Lane PC: Positive control; Lane NC- Negative control.

The amplified DNA fragment of six isolates were cloned and sequenced. The sequenced region contained 1050 to 1055 bp in different isolates. Of this, coat protein (CP) gene consisted of 816-822 nucleotides, potentially coding for 272-274 amino acid residues in different isolates. Sequences were deposited at the GenBank and their accession number is listed in Table 4. The coat protein gene sequences of the isolates were compared among themselves and also with corresponding CP genes from other CdMV isolates available in the GenBank (Table 4).

Table 4: Geographical origin and GenBank accession numbers of CdMV isolates		
Designation	**Region (District, State)**	**Accession No.**
APG-1	Appangala (Kodagu, Karnataka)	JN544081 (This study)
SKP	Sakleshpur (Hassan, Karnataka)	JN544082 (This study)
SRS	Sirsi (Uttara Kannada, Karnataka)	JN544079 (This study)
PMP	Pampadumpara (Idukki, Kerala)	JN544077 (This study)
MPD	Meppadi (Wayanad, Kerala)	JN544080 (This study)
TDS	Thadiyankudisai (Dindigul, Tamil Nadu)	JN544078 (This study)
SMR	Somvarpet (Kodagu, Karntaka)	AY609386
MRD	Margodu (Kodagu, Karnataka)	AY609385
ANM	Anemahal (Hassan, Karnataka)	AY833735
LKP	Lakshmipura (Hassan, Karnataka)	AY823986
YSR	Yeslur (Kodagu, Karnataka)	AF189125
KTP	Kattappana (Idukki, Kerala)	AJ312774
VDR	Vandiperiyar (Idukki, Kerala)	AJ308477
KSR	Kursupara (Idukki, Kerala)	AJ308476
APG-2	Appangala (Kodagu, Karnataka)	AJ308472
TLM	Thalathamane (Kodagu, Karnataka)	AJ308475
MDK	Madikeri (Kodagu, Karnataka)	AJ308474

The sequence identity of CP gene among six isolates used in the present study ranged from 75.3 – 96.9% and 82.4 - 98.1% at nucleotide and amino acid level respectively while, identity with other 11 isolates varied from 74.8 - 99.3% and 80.9 - 99.2%. When nucleotide sequences of CP gene from all CdMV isolates were compared, variability ranged from 0.7-25.2% at nucleotide and 0.8-19.1% at amino acid level while, identity with other species of *Macluravirus* was < 62%. Among other distinct species of the *Macluravirus*, CdMV was closer to *Alpinia mosaic virus*. The deduced amino acid sequence of CP gene showed high level of identity in the core and C-terminal region, whereas the first 39 amino acids from the N-terminus end showed considerable variations. Differences of 6 – 49 amino acids were observed in the CP among isolates. A stretch of amino acid residues (WCANNGTSSE) which is highly conserved in the genus *Macluravirus* was also found in all the CdMV isolates (position 122- 131). One amino acid deletion was found in six isolates (MDK, SKP, APG-2, SMR, TLM and LKP) at 38^{th} position and two amino acid deletions were observed at 38^{th} and 39^{th} positions in four isolates (MRD, YSR, APG-1 and ANM).

Based on percent identities among strains, they could be classified into three groups (Table 5). All isolates of Karnataka (except Sirsi) belonged to one group with an identity ranging from 90 to 99.2 % while, the all isolates of Kerala along with one isolate from Tamil Nadu belonged to another group with identity ranging from 87.1 - 98.9 %.

Table 5: Grouping of CdMV isolates based on percent identities in the nucleotide (values shown in brackets) and deduced amino acid of coat protein gene

Groups and isolates	Percent identity among groups		
	Group 1	**Group 2**	**Group 3**
Group I MRD, YSR, APG-1, ANM, MDK, APG-2, SMR, TLM, LKP, SKP	90.0 - 99.2 (82.2 - 99.3)	83.5- 86.8 (76.1 - 77.8)	80.9 –86.0 (74.8 – 77.6)
Group II SRS		100	83.5 – 85.7 (76.1 - 77.8)
Group III KTP, VDR, PMP, MPD, KSR, TDS			87.1 - 98.9 (82.8 - 97.4)

The results of multiple alignments of 17 CdMV isolates based on amino acid sequences of CP were used to generate a phylogram illustrating their phylogenetic relationship. Scrutiny of isolates revealed that, all the six isolates of group III (five isolates from Kerala and one isolate from Tamil Nadu) clustered together (Table 5; Fig 1). On the other hand, isolates from Karnataka showed more divergence. Of the ten isolates belonging to group I from Karnataka, three isolates with >98% identities showed close clustering that were slightly away from remaining seven isolates. One isolate belonging to group II (SRS) showed distant relationship with both the other groups (I and II) thus clearly indicating its distinctiveness.

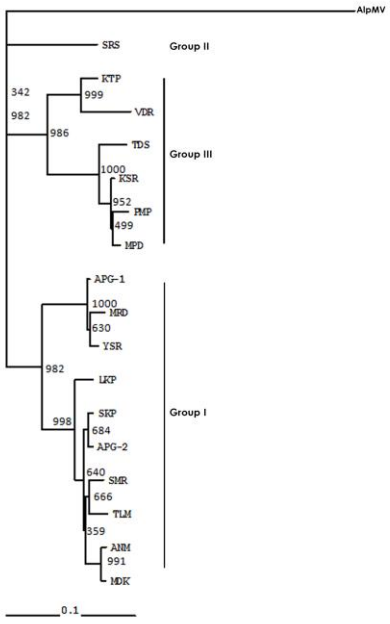


Fig. 1: Phylogram illustrating phylogenetic relationship based on the multiple alignment of the coat protein amino acid sequences of 17 CdMV isolates. *Alpinia mosaic virus* (AlpMV) was taken as outgroup. Bootstrap values are shown at the nodes.

Seed transmission studies of CdMV

Seed transmission studies were carried out using immature capsules, immature and mature seeds collected from virus infected plants of the cv. Njallani Green Gold. In RT-PCR, all the samples showed a clear amplification of 1050 bp for CdMV, indicating the presence of the virus in immature capsules and immature and mature seeds tested (Table 6). Grow-out test was also carried out with the seeds collected from infected capsules. The seeds were sown in separate seed pans and kept in insect proof glass house. All the seedlings were asymptomatic and the absence of both the viruses in the seedlings was confirmed by performing RT-PCR (Table 7).

Table 6: Testing seeds and capsules from infected plants for CdMV by RT-PCR						
Capsules/ seeds tested	No. of Capsules/ seeds tested		No. of Capsules/ seeds positive in RT-PCR		% of infection	
	CdMV	Healthy	CdMV	Healthy	CdMV	Healthy
Immature capsules	14	7	14	0	100	0
Immature seeds	35	7	35	0	100	0
Mature seeds	35	7	35	0	100	0

Table 7: Details of seed transmission study of CdMV						
No. of seeds sown	No. of seeds germinated	Days taken for germination	% of germination	Symptomatic seedlings	No. of seedlings tested	No. of PCR $+^{ve}$ seedlings
300 (from infected source)	245	21-26 days	81.7	0	70	0
100 (Healthy)	82	21-26 days	82.0	0	10	0

(III) Purification, production of polyclonal antiserum and ELISA based detection of CdMV

Purification of CdMV

Protocol for purifying the causal virus (*Cardamom mosaic virus*) of *katte*/mosaic disease of cardamom was standardized. The standardized protocol is as follows:

The leaves expressing typical mosaic symptoms along with the midrib tissues were ground in 3 volumes of 0.5 M potassium phosphate buffer (pH 7.1) containing 0.01 M EDTA and 0.1% β-mercaptoethanol. The ground material was strained through double layered muslin cloth. The resultant debris was re-extracted with 1 volume of 0.5 M potassium phosphate buffer (pH 7.1) containing 0.01 M EDTA and 0.1% β-mercaptoethanol and to this extract 1% Triton X 100 was added at room temperature. The extract was stirred using a magnetic stirrer at room temperature for 15 minutes and subsequently centrifuged at 10,000 g for 10 minutes at 10^0C in Beckman Coulter JA – 25.5 centrifuge. The supernatant was collected in fresh tubes and ultracentrifuged at 78,000 g for 2 hours at 4^0C in Beckman Optima^{TM} LE – 80K Ultracentrifuge. The pellet thus obtained was dissolved in 0.025 M potassium phosphate (pH 7.1) containing 1 mM EDTA by stirring for 30 minutes at 4^0C followed by centrifuging at 8,000 g for 10 minutes at 4^0C. The supernatant was collected and layered over 10 ml of sucrose

gradients i.e., 10%, 20%, 30% and 40%, prepared in 0.025 M potassium phosphate (pH 7.1) containing 1 mM EDTA. This was followed by ultracentrifugation at 78,000 g for 2.5 hours at 4^0C. The resultant pellet was dissolved in 100 µl distilled water and stored at -20^0C for immunizing rabbits and producing polyclonal antiserum.

Production of polyclonal antiserum against CdMV

A New Zealand white rabbit was sensitized by injecting purified virus mixed with Freund's complete adjuvant (1:1 ratio) into both hip muscles. Subsequent injections were given at 8, 15, 22 and 29 days after the initial injection. One week after the last injection, about 10 ml of blood was collected by bleeding marginal vein of the ear. The blood was allowed to clot at room temperature for one hour followed by incubation at 4ºC overnight to separate the serum. Using a sterile micro pipette, the separated serum was collected into a sterile collection vial and clarified by centrifuging at 5000 rpm for 1 minute. The purified serum was mixed with equal volume of 40% glycerol and stored at -20 ºC for further use.

Detection of CdMV by direct antigen coated ELISA (DAC-ELISA)

DAC – ELISA was performed using healthy and infected cardamom leaf samples. The tissue was ground in coating buffer containing 2% PVP in the ratio 1:10 (tissue: coating buffer). Three different dilutions (1:250, 1:500, 1:1000) of CdMV polyclonal antiserum was cross-adsorbed with healthy cardamom sap and used to load into the ELISA plate. Alkaline phosphatase labeled goat anti rabbit antibody was used at 1:7500 dilution. Absorbance was recorded after 1 hour of substrate addition. Of the three different dilutions of polyclonal antiserum (1:250, 1:500, 1:1000) tested, 1:250 dilution gave higher absorbance compared to the other dilutions (Table 8). The other dilutions *viz.* 1:500 and 1:1000 did not produce significant reaction as compared to the healthy control.

Table 8: ELISA based detection of CdMV

CdMV isolates	A_{405} Values
Sirsi, Uttara Kannada	1.2
Kyanahally, Sakleshpur	1.1
Bobbanahally, Sakleshpur	3.0
Appangala-1, Kodagu	1.2
Appangala-2, Kodagu	1.4
Healthy	0.34

(IV) Development of Reverse Transcription- Polymerase Chain Reaction (RT-PCR) and real time- PCR based detection of CdMV

Development of Reverse Transcription- Polymerase Chain Reaction (RT-PCR) and validation

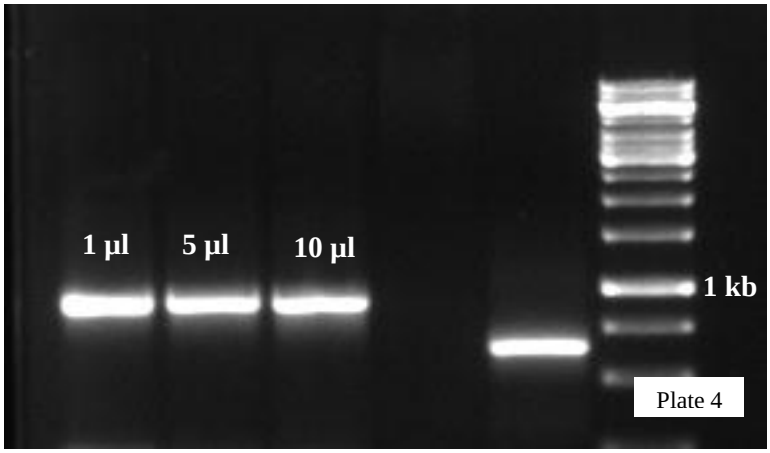
Protocols for RNA isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for detecting CdMV from the leaves obtained from katte affected cardamom plants were standardized.

The standardized protocols for RNA isolation and RT- PCR conditions are as follows:

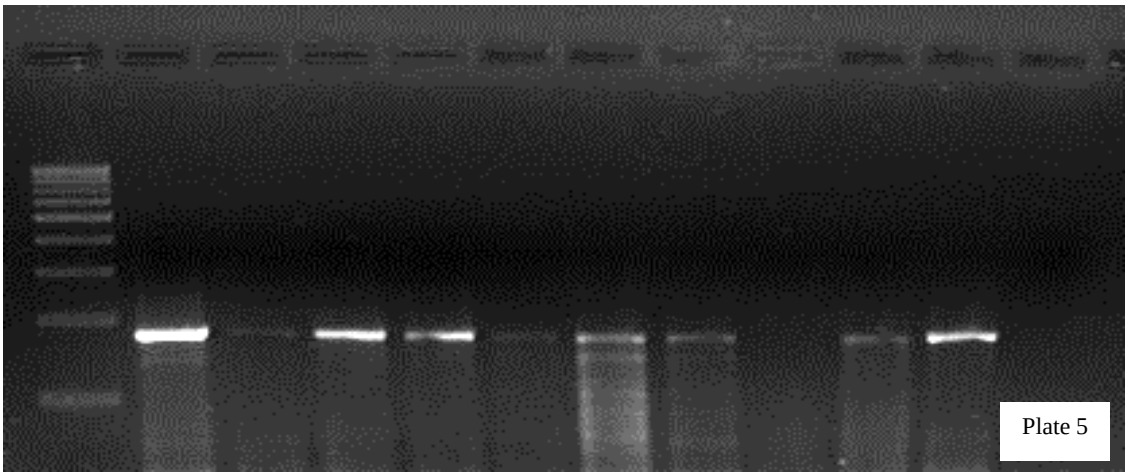
For total RNA isolation, 50 mg of tender cardamom leaf tissue with characteristic mosaic symptoms was ground in 500 µl of denaturing solution [4 M guanidium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% (w/v) sodium N-lauryl sarcosine, 0.1% (v/v) β-mercaptoethanol] and collected in a microcentrifuge tube. To this, 50 µl of 2 M sodium acetate (pH 4.0), 500 µl of water-saturated phenol and 100 µl of chloroform – isoamyl alcohol (49:1) were serially added with intermittent mixing at each step. The tube was then shaken for 15 seconds and kept on ice for 15 minutes. The resultant homogenate was centrifuged at 12,000 g for 15 minutes at 4^0C. The aqueous phase was carefully collected without getting mixed with the organic and interphases in a fresh tube and mixed with equal volume of isopropanol followed by incubation at -80^0C for 1 hour for the precipitation of total RNA. Centrifugation at 12,000 g for 10 minutes at 4^0C yielded a pellet which was washed with 75% ethanol and air dried. The final pellet was dissolved in 80 µl of HPLC grade water and stored at -20^0C for further processing. Synthesis of cDNA was performed at 42^0C for 45 minutes and the amplification was carried out for 40 cycles in an automated thermal cycler (Eppendorf Master Cycler) with the primer combination, AIB 75 (forward) (CACCGCTTGCACCAATGAC) and AIB 44 (reverse) (GAAAACCCACAAAAACTCCC). The expected size of the amplicons was 1 kb. Each cycle consisted of denaturation at 94^0C (30 seconds), primer annealing at 50^0C (1 minute) and extension at 72^0C (10 minutes), followed by final extension at 72^0C (10 minutes). The amplified products were analyzed with 500 bp DNA ladder (Genei) on 1% agarose gel containing ethidium bromide. The bands were visualized and photographed using an UV transilluminator and a gel documentation apparatus. The result obtained

from standardized protocol was also compared with that of RNA isolation kit at different template concentrations.

The virus could be detected at 1, 5 and 10µl template concentrations using RNA isolation kit (Plate 4) while the standardized protocol could detect the virus at 2 and 7 µl template concentrations from the infected leaf samples (Plate 5).



Lane 1: Appangala (1µl) , Lane 2: Appangala (5µl) , Lane 3: Appangala (10µl) , Lane 4: Appangala (20µl) , Lane 5: Positive control (CMV infecting black pepper), Lane 6: Marker



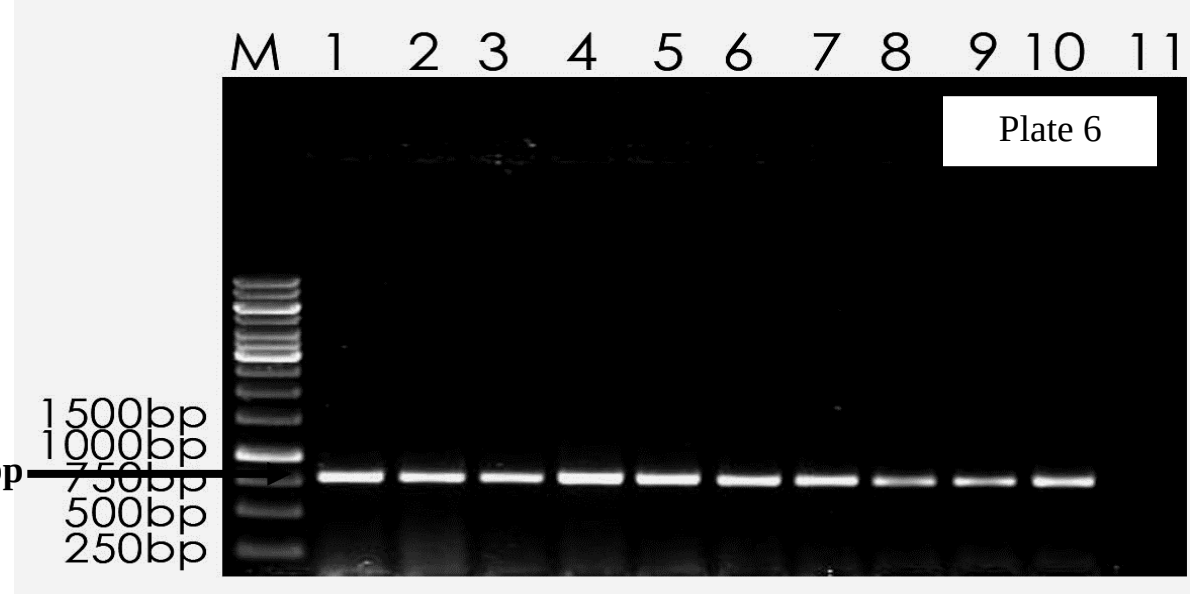
Lane 1: Marker, Lane 2: Appangala, Lane 3: Kundally, Lane 4: Nagarahally, Lane 5: Kakkabe, Lane 6: Kerahally, Lane 7: Bobbanahally, Lane 8: Kattappana, Lane 9: Myladumpara I, Lane 10: Myladumpara II, Lane 11: Mankulam, Lane 12: Healthy control

Validation of RT-PCR based protocol for the detection of CdMV by testing field samples of cardamom

Protocols standardized for RNA isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for detecting *Cardamom mosaic* were validated by testing 53 cardamom samples collected from Karnataka and Kerala (Table 9 and Plate 6).

Table 9: Reverse Transcription – Polymerase Chain reaction (RT-PCR) based detection of CdMV from field samples

State	District	No. of samples tested	No. Positive in RT-PCR
Karnataka	Kodagu	29	14
	Hassan	13	12
Kerala	Wayanad	2	1
	Idukki	9	8



Lane M: DNA molecular size markers; Lane 1–9: Cardamom samples from different regions; Lane 10: Positive control (known infected plant); Lane 11: Negative control (known healthy plant)

Real time- PCR based detection of CdMV

The specificity of SYBR green assay was evaluated using three different reactions, which included infected sample, healthy (negative) control and water (without

template) control. In order to check the influence of RNA extraction method, real time assay was performed with RNA extracted by manual and kit methods separately.

The real time PCR products were analyzed by agarose gel electrophoresis. The assay with CdMV sample displayed the expected band of 127 bp (CdMV) whereas healthy and water control did not amplify (Plate 7).

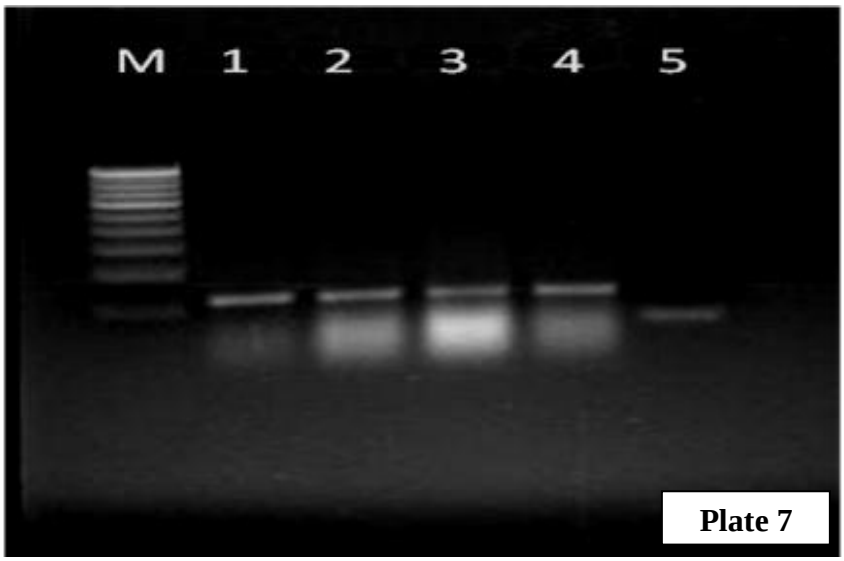


Plate 7

Lane M - 100bp ladder; Lane 1-BBrMV isolate from Appangala, Lane 2- BBrMV isolate from Idukki, Lane 3- CdMV isolate from Appangala, Lane 4- CdMV isolate from Idukki, Lane 5-Healthy control (using BBrMV specific primers).

The sensitivity of SYBR green assay was tested using different template RNA volumes *viz.* 0.01, 0.1, 1.0, 3.0 and 5.0 µl template for RNA isolated by manual method and 0.005, 0.01, 0.1, 1.0, 3.0, 5.0, 7.0 and 10.0 µl template for RNA isolated by kit method. For manual method, in the case of CdMV, the SYBR green RT-PCR was unable to detect the virus at a template volume below 1 µl while virus could be detected in template volumes ranging from 1 to 5 µl with Ct values from 26.0 to 20.0 (Table 10 and Fig. 2).

Table 10: Ct values of CdMV detection in real time RT-PCR using the templates isolated by manual method	
RNA template volume	Ct values of CdMV
0.01 µl	-
0.1 µl	-
1.0 µl	26.0
3.0 µl	22.90
5.0 µl	20.0

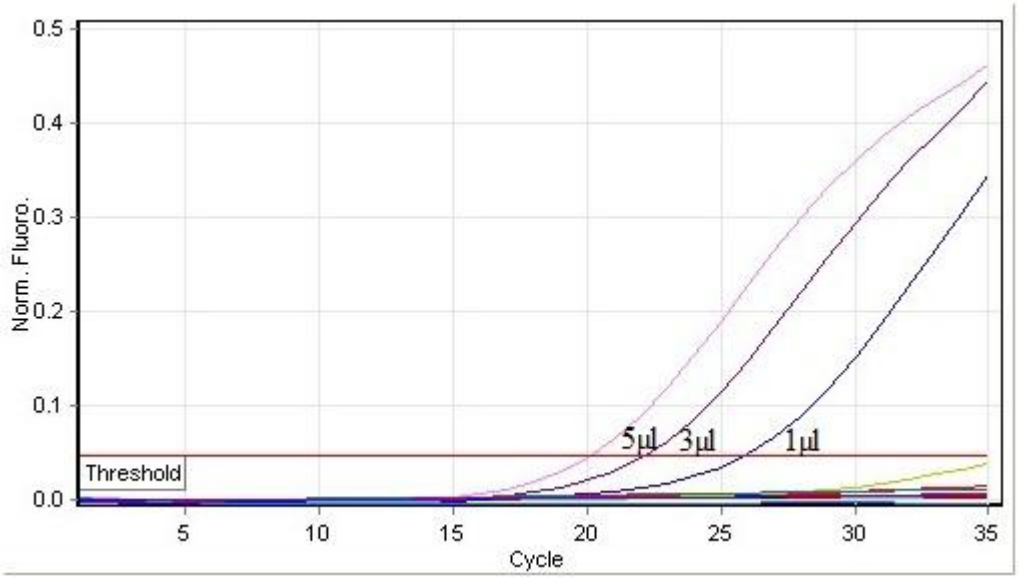


Fig. 2: Real time RT-PCR detection of CdMV infected cardamom sample with manual method. Amplification plots show the sensitive threshold as 1 µl with a Ct value of 26.0. The water control and healthy control did not result any signals.

For kit method, in the case of CdMV, the SYBR green RT-PCR was unable to detect the virus at a template concentration below 0.1 µl (Table 11 and Fig 3). However, the real time assay with template isolated by manual method could detect the virus at a minimum concentration of 1 µl. This indicates that, the sensitivity of SYBR green RT-PCR could be increased by using template isolated by kit method.

Table 11: Ct values of CdMV detection in real time RT-PCR using the template isolated by kit method	
RNA template volume	**Ct values of CdMV**
0.005 µl	-
0.01 µl	-
0.1 µl	27.62
1.0 µl	19.86
3.0 µl	19.17
5.0 µl	18.76
7.0 µl	19.24
10.0 µl	22.11

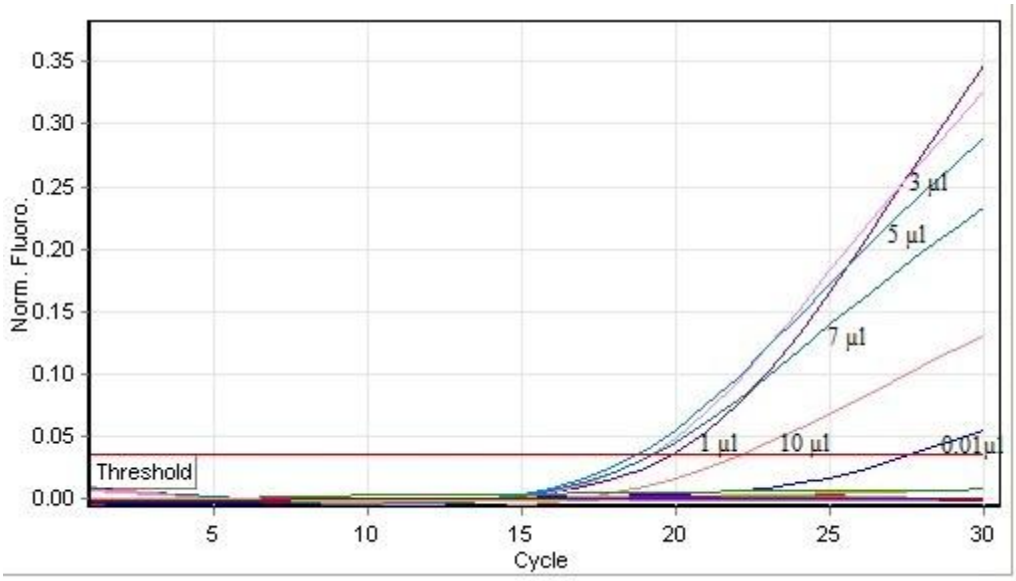


Fig. 3: Real time RT-PCR detection of CdMV infected cardamom sample using the templates isolated by kit method. Amplification plots show the sensitive threshold as 0.01 µl with a Ct value of 27.62. The 0.005 µl, 0.01 µl template, water control and healthy control did not result any signals.

Chlorotic streak disease (*Banana bract mosaic virus*)

(I) Survey, incidence and symptomatology

Survey and incidence of chlorotic streak disease

During surveys conducted to study the incidence and distribution of mosaic (*Katte*/*marble*) disease caused by CdMV in cardamom plantations, a new kind of viral disease exhibiting chlorotic streak symptoms on veins were observed in certain

plantations. Based on the symptoms, the disease was named as chlorotic streak. Of the 77 plantations in 49 locations surveyed, incidence of the disease ranged from 0-15%. In Kerala, of the 44 plantations belonging to 30 locations surveyed, the disease incidence ranged from 0- 15%. The highest incidence of 15% was recorded at Vythiri Taluk of Wayanad District while no incidence was recorded in Palakkad District. In Karnataka, incidence ranged from 0-5% with highest incidence (5%) in Sirsi, Siddapur and Yellappur Taluks of Uttara Kannada District while no disease was recorded in Madikeri and Virajpet Taluks of Kodagu District, and Sakleshpur Taluk of Hassan District. Similarly, in Tamil Nadu chlorotic streak was not noticed in any of the plantations surveyed (Table 12).

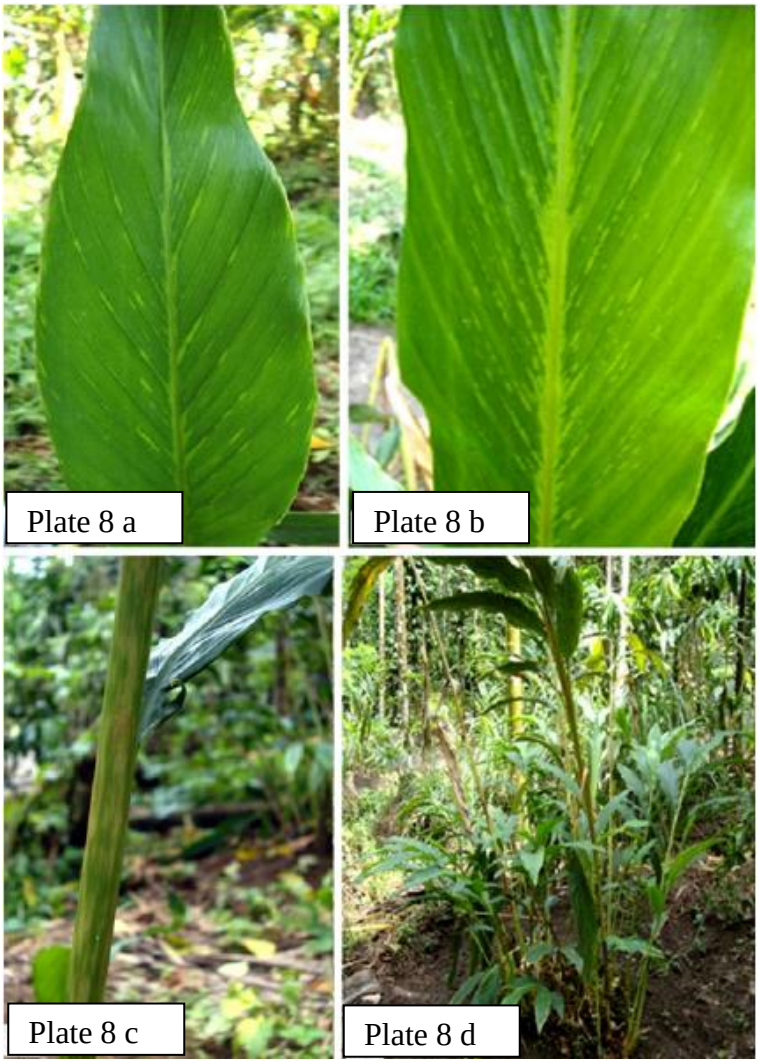
Symptomatology of chlorotic streak disease

The disease was characterized by continuous or discontinuous spindle shaped yellow or light green intravenous streaks along the veins and midrib (Plate 8 a). These streaks later coalesce together and imparted yellow or light green colour to the veins (Plate 8 b). Discontinuous spindle shaped mottling on pseudostem and petioles were also

Table 12: Distribution and incidence of chlorotic streak disease in major cardamom growing regions of India

State/ District	Taluk	No of locations surveyed	No. of fields surveyed	Disease incidence	
				Range %	Mean
Karnataka					
Uttara Kannada	Sirsi	3	4	1-5	2.75
	Siddapur	1	3	0-5	1.6
	Yellapur	3	3	1-5	2.0
Kodagu	Madikeri	3	7	0	0.0
	Somwarpet	2	4	0-4	1.0
	Virajpet	1	2	0	0.0
Hassan	Sakleshpur	3	3	0	0.0
Kerala					
Idukki	Udumbanchola	16	24	0-12	2.13
	Peermade	4	4	0-6	1.75
	Devikulam	5	6	2-12	2.8
Wayanad	Vythiri	3	6	0-15	7.5
Palakkad	Chittoor	2	4	0	0.0
Tamil Nadu					
Coimbatore	Valparai	1	4	0	0.0
Dindigul	Kodaikkanal	1	2	0	0.0
Salem	Yercaud	1	1	0	0.0

noticed (Plate 8 c). In severe cases, tillering in infected plant was suppressed (Plate 8 d). The distinguishing feature of the present disease was formation of intravenous chlorotic streaks; hence the disease was named as chlorotic streak.



Symptomatological difference between two isolates of BBrMV *viz*., Sirsi of Karnataka and Upputhara, Idukki districts of Kerala are presented in Table 13.

Table 13:		Symptomatology of BBrMV infection in cardamom	
		Locations	
		Upputahara (Idukki isolate)	Sirsi (Karnataka isolate)
Leaf	Young	Discontinuous or continuous spindle shaped streaks along the veins and chlorotic patches on the leaves	Veins turn light green and appearance of spindle shaped streaks in the inter-veinal areas
	Mature	Continuous light green or light yellow streaks along the midrib and light green coloured veins	Continuous light green or light yellow streaks along the midribs and vein thickening
Pseudostem		Discontinuous mottling along the pseudostem and petioles	Discontinuous and continuous mottling
Capsules		Normal capsules, less yield	Normal capsules, less yield

(II) Identification, characterization of BBrMV isolates based on coat protein gene sequencing and transmission studies

Detection of BBrMV in cardamom by electron microscopy

Electron microscopy of leaf dip preparations negatively stained with 2% uranyl acetate (pH 4.5) was carried out using JEOL 100 CFII transmission electron microscope at the Unit of Plant Virology, Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi. Flexuous BBrMV particles were observed in the leaf dip preparations of infected leaf samples (Plate 9).

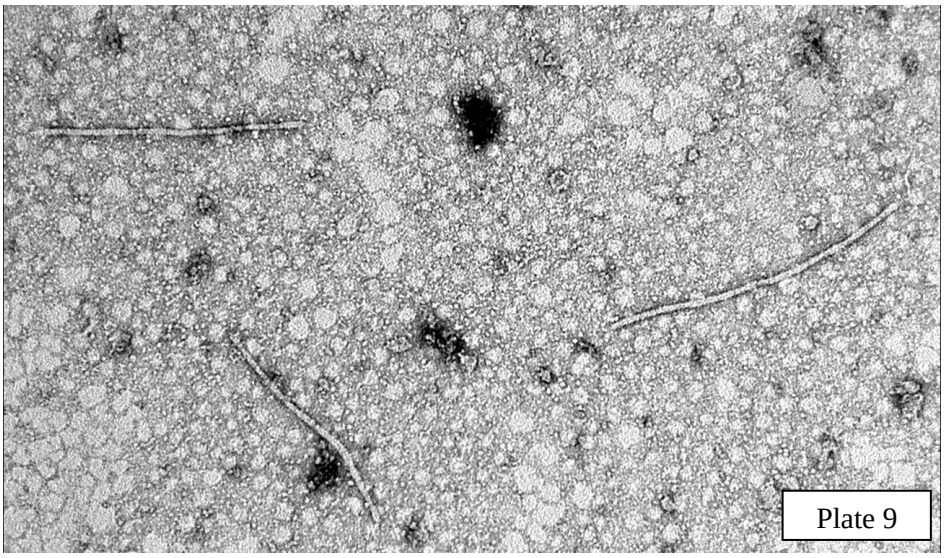


Plate 9

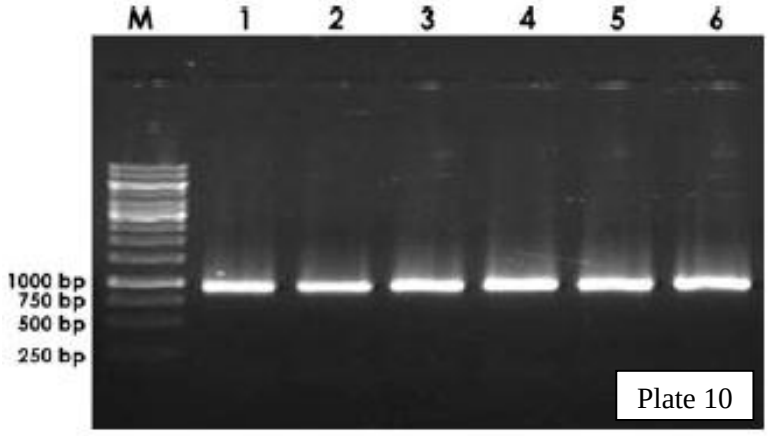
Characterization of BBrMV infecting cardamom - Cloning, sequencing and sequence analyses

The plants infected with chlorotic streak disease tested negative in RT-PCR for CdMV. Leaf dip electron microscopy of these samples showed the presence of flexuous virions resembling *Potyvirus*. In view of this, total RNA extracted from these plants were subjected to RT-PCR using primers targeting the conserved region of Potyvirus. Potyvirus genus specific primers for the conserved 'WCIEN' and Poly (A) region were synthesized as per Pappu *et al*. (1993). To amplify the coat protein (CP) gene of BBrMV, forward and reverse primers were designed to the conserved region identified based on multiple sequence alignment of coat protein gene and 3´ untranslated region (UTR) of all available BBrMV sequences from GenBank database. The forward primer (5´ TCTGGAACGGAGTCAAC 3´) represented first 17 bases of the CP gene and reverse primer (5´ GCACACATAATTATAGGGAG 3´) was derived from the 3´ untranslated region (UTR). Total RNA was isolated from 100 mg of leaf tissue following the method described by Siju *et al.* (2007). The RT-PCR profile using *Potyvirus* genus specific primers consisted of one cycle at 42^0C for 45 min for cDNA synthesis followed by 35 cycles reaction profile involving 30 s of denaturation at 94^0C, 2 min of annealing at 42^0C and 1 min of extension at 72^0C and a single cycle of final extension at 72^0C for 10 min. The RT-PCR profile for the BBrMV specific primers were same as above except that annealing was carried out at 52^0C for 1 min. The reaction products were analysed on 0.8% agarose gel along with 1kb DNA ladder. The DNA bands were visualized and photographed using a UV transilluminator and a gel documentation apparatus (Cell Biosciences, Santa Clara, USA).

The PCR product thus obtained was eluted from the gel using GenElute Gel Elution kit (Sigma-Aldrich, Bangalore, India), cloned into pTZ57R/T cloning vector (Fermentas, Glen Burnie, USA) and transformed into competent *E. coli* strain DH5α using InsTAclone PCR cloning Kit (Fermentas, Glen Burnie, USA) following manufacturer's instructions. Recombinant clones were identified by PCR as well as restriction endonuclease digestion and selected clones were sequenced from both the directions at the automated DNA sequencing facility available at Chromous Biotech,

Bangalore, India. Sequence data were compiled using Seqaid Version 3.6 (Rhoads and Roufa, 1985). Multiple sequence alignments were made using ClustalX (1.81) and trees were generated using TREEVIEW software. Percent identities were determined using Bioedit program version 5.0.9. The coat protein nucleotide and amino acid sequences of other BBrMV isolates used for comparison were obtained from GenBank (Benson *et al.*, 1999). The BLAST programme (Altschul *et al.*, 1997) was used to identify related sequences available from GenBank database.

Sequencing and BLAST analysis of this fragment showed *Banana bract mosaic virus* (BBrMV) as the closest relative. RT-PCR performed with BBrMV specific primers resulted in an amplicon of ~950bp in all five samples collected from different geographical regions while, no product was observed in healthy control (Plate 10).



Lane M: Marker (1 kb ladder); Lanes 1 -5: chlorotic streak infected cardamom samples from five different geographical areas; Lane 6: positive control (BBrMV infected banana sample)

All these samples tested negative when CdMV specific primers were used in RT-PCR. The amplified product from all five isolates was cloned and sequenced. Sequenced region contained 945 nucleotides in all five isolates in which, first 900 bp represented coat protein region potentially coding for 300 amino acid residues, while remaining 45 bases represented 3' UTR. The sequences were deposited in GenBank and their accession numbers is provided in Table 14.

Table 14: Host, geographical origins and GenBank accession numbers of the BBrMV isolates used in this study

Designation	Country	Host	Accession No.
Card-1	India (Madikeri, Karnataka)	Cardamom	HQ709165
Card-2	India (Mudigere, Karnataka)	Cardamom	HQ709166
Card-3	India (Sirsi, Karnataka)	Cardamom	HQ709164
Card-4	India (Sirsi, Karnataka)	Cardamom	AY776327
Card-5	India (Idukki, Kerala)	Cardamom	HQ709163
Card-6	India (Wayanad, Kerala)	Cardamom	HQ709162
Ban-1	India (Coimbatore, Tamil Nadu)	Banana	AF071582
Ban-2	India (Coimbatore, Tamil Nadu)	Banana	AF071583
Ban-3	India (Coimbatore, Tamil Nadu)	Banana	AY494979
Ban-4	India (Thiruchirapalli, Tamil Nadu)	Banana	AF071584
Ban-5	India (Thiruchirapalli, Tamil Nadu)	Banana	EU009210
Ban-6	India (West Godavari, Andhra Pradesh)	Banana	AY953427
Ban-7	India (Karnataka)	Banana	EF654655
Ban-8	India	Banana	EU699770
Ban-9	Philippines (Los Banos)	Banana	AF071590
Ban-10	Philippines (Los Banos)	Banana	AF071585
Ban-11	Philippines (Davao)	Banana	AF071586
Ban-12	Philippines	Banana	DQ851496
Ban-13	Philippines	Banana	EU414267
Ban-14	Thailand	Banana	AF071589
Ban-15	Vietnam (Hau)	Banana	AF071588
Ban-16	Western Samoa (Nu'u)	Banana	AF071587

The CP gene sequence of isolates were compared among themselves and with corresponding CP gene sequences of all available BBrMV isolates (Table 14) and a few other potyviruses. Nucleotide sequence identity among cardamom isolates sequenced in the present study varied from 97- 99% and 96 - 99% at nucleotide and amino acid levels, respectively. Sequence analysis with all available BBrMV isolates infecting banana and cardamom showed an identity ranging from 94-100% and 95-100% at nucleotide and amino acid sequence levels, respectively (Table 15).

Table 15: Percent identity of the nucleotide (above the diagonal line) and amino acid (below the diagonal line) in the coat protein region among BBrMV isolates infecting cardamom and banana

Sequence	Cardd-1	Cardd-2	Cardd-3	Cardd-4	Cardd-5	Cardd-6	Ban-1	Ban-2	Ban-3	Ban-4	Ban-5	Ban-6	Ban-7	Ban-8	Ban-9	Ban-10	Ban-11	Ban-12	Ban-13	Ban-14	Ban-15	Ban-16
Card-1		98	98	97	98	98	98	97	98	95	98	97	97	95	95	95	95	96	95	95	95	94
Card-2	97		98	97	98	97	98	97	98	95	98	97	97	95	94	95	95	95	95	95	95	94
Card-3	99	98		98	98	99	99	98	98	96	99	98	98	96	95	96	96	97	96	96	96	96
Card-4	99	97	99		97	98	98	97	98	96	98	97	97	97	96	96	97	98	97	96	96	96
Card-5	97	96	98	97		98	98	97	98	95	98	97	97	96	94	94	95	95	95	95	95	94
Card-6	98	97	99	99	97		98	98	99	96	98	98	98	96	95	95	96	96	96	96	96	95
Ban-1	98	97	99	99	97	98		98	99	96	98	98	98	96	95	95	96	96	96	96	96	95
Ban-2	97	96	98	98	96	97	98		98	95	98	97	97	95	94	95	95	95	95	95	95	94
Ban-3	98	97	99	99	97	98	99	98		95	98	98	98	96	95	95	96	96	96	95	96	95
Ban-4	97	96	98	99	96	98	98	97	98		96	95	95	97	98	98	97	98	97	99	100	97
Ban-5	98	97	99	100	97	99	99	98	99	98		97	98	96	95	95	96	96	96	96	96	95
Ban-6	98	96	99	98	97	98	98	97	98	97	98		97	95	94	94	95	95	95	95	95	94
Ban-7	98	96	99	99	97	98	99	97	99	97	98	97		95	94	95	95	96	95	95	95	94
Ban-8	98	96	99	99	97	98	99	97	99	99	98	97	98		96	97	100	99	100	97	97	97
Ban-9	97	95	97	98	95	97	97	96	97	98	98	96	97	98		97	96	97	96	98	98	96
Ban-10	97	95	97	99	95	97	97	96	97	98	98	96	96	98	97		97	97	97	98	98	96
Ban-11	98	96	99	99	97	98	99	97	99	99	99	97	98	100	98	98		99	100	97	98	97
Ban-12	98	96	99	99	97	98	99	97	99	99	99	97	99	99	98	98	98		99	98	98	97
Ban-13	98	96	99	99	97	98	99	97	99	99	99	97	98	100	98	98	100	99		97	97	97
Ban-14	98	96	98	99	96	98	98	97	98	100	99	97	98	99	98	98	99	99	99		99	97
Ban-15	97	96	98	99	96	98	98	97	98	99	98	97	97	99	98	98	99	99	99	100		98
Ban-16	96	95	97	98	95	97	97	96	97	97	97	96	98	97	97	96	98	97	98	98	98	

Identity within banana isolates of BBrMV ranged from 94-100% and 96-100% at nucleotide and amino acid levels, respectively. Most of the differences were seen in N-terminal region of coat protein. Six BBrMV banana isolates from India showed high identity towards to BBrMV cardamom isolates (97-99%) while, BBrMV banana isolates from Philippines, Thailand, Vietnam, Western Samoa along with two Indian isolates showed identity of 94-96.8% with cardamom isolates. BLAST analysis showed *Sugarcane mosaic virus* (SCMV) as the closest species to the present virus. Identity in the CP gene sequence of SCMV with present virus isolates was 60% at nucleotide and 55% amino acid levels. The identity in the CP gene with CdMV (genus: *Macluravirus*) infecting cardamom was only 23% at nucleotide and amino acid levels, thus clearly indicating their difference at the genetic level. High CP sequence identity of present isolates (>94%) with BBrMV clearly indicates that, the causal virus associated with chlorotic streak disease is a strain of BBrMV.

Multiple sequence alignment of CP gene deduced amino acid revealed a slightly variable N-terminal region that had maximum sequence heterogeneity while, the core and C- terminus was found to be highly conserved. Differences of two to six amino acids were observed among cardamom isolates. Analysis of the sequence revealed several motifs commonly found in potyviral coat proteins including a DAG amino acid triplet, a WCIEN box in the core region and an RQ, AFDF and QMKAA. Phylogenetic tree was

constructed using nucleotide sequences of the entire coat protein coding region of 22 BBrMV isolates (Fig. 4).

Phylogenetic analyses of the BBrMV isolates revealed two major clusters, one with all cardamom along with six banana isolates from India while, the other cluster consisted of banana isolates from Philippines, Thailand, Vietnam, Western Samoa and two Indian isolates.

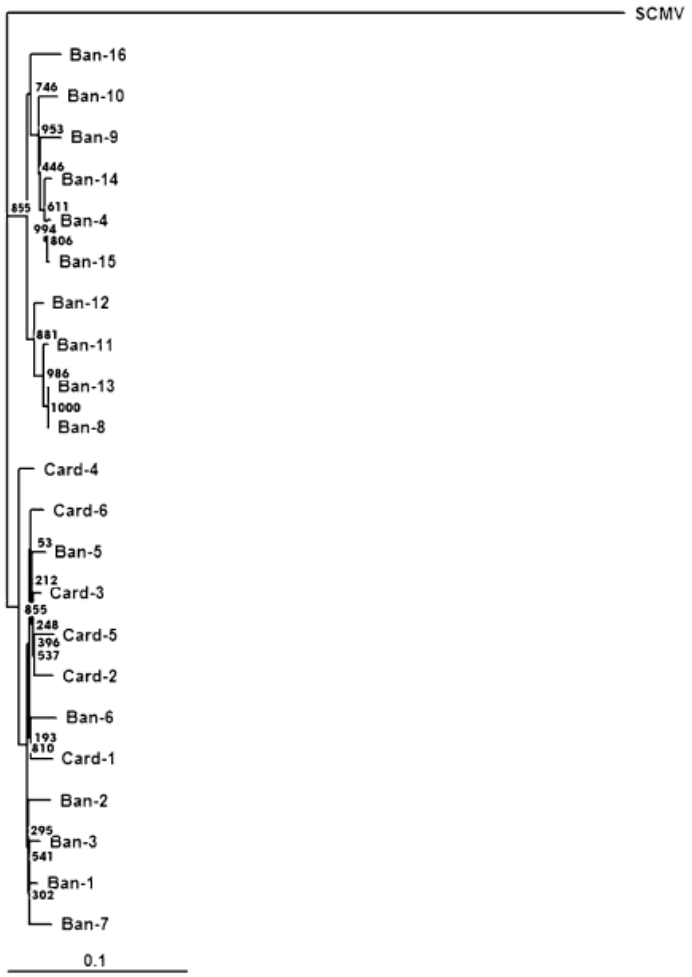


Fig. 4: Phylogram drawn by Neighborhood Joining method in Clustal X (1.81), illustrating phylogenetic relationships based on the multiple alignments of nucleotide sequences of 22 distinct isolates of BBrMV infecting banana and cardamom. *Sugar cane mosaic virus* (SCMV) was used as outgroup. The bootstrap values are shown at the individual nodes.

Transmission studies of BBrMV using aphids and through sap inoculation

Transmission studies of BBrMV through aphids and by sap inoculation were carried out. For this experiment, Appangala isolate of BBrMV infecting banana and Wayanad isolate of BBrMV infecting cardamom were used. Tissue cultured plants of banana (8 to one year old) and one-year-old seedling of IISR Kodagu Suvasini were used as host plants.

Aphid transmission:

For aphid transmission study, *Pentalonia caladii* (formerly *Pentalonia nigronervosa* f. *caladii*) was used as the vector and non-persistent mode of transmission was employed. Leaves exhibiting symptoms characteristic to BBrMV infection were collected from banana and cardamom were used for acquisition of the virus. Transmission experiments were designed in all possible combinations *viz*., banana isolate of BBrMV to banana, banana isolate of BBrMV to cardamom, cardamom isolate of BBrMV to banana and cardamom isolate of BBrMV to cardamom. Two rounds of inoculations were carried out with the viruliferous vector. In banana to banana and banana to cardamom combination, six plants each of banana and cardamom were used. While, in cardamom to cardamom to cardamom and cardamom to banana combination, five plants each of cardamom and banana were used. Second round of inoculation was taken up three weeks after first inoculation. The inoculated plants were kept under glass house conditions and observed for the development of symptoms (if any). The inoculated plants (in all combinations) did not develop any visible symptoms characteristic to BBrMV infection even four months after the second inoculation. Subsequently the samples originating from the inoculated plants were subjected to real time-PCR and the absence of the BBrMV was confirmed in all the samples tested indicating lack of transmission of the virus by aphid.

Mechanical (sap) transmission:

For sap transmission, young leaves exhibiting symptoms characteristic to BBrMV infection (both from banana and cardamom) were collected and homogenized in 0.1 M phosphate buffer (pH 7.0). Carborundum was used as abrasive and the

transmission was carried out by following standard inoculation procedures. Similar to aphid transmission, sap inoculation was also carried out in all possible combinations of the hosts. For this experiment, Appangala isolate of BBrMV infecting banana and Wayanad isolate of BBrMV infecting cardamom were used. Tissue cultured plants of banana (8 to one year old) and one-year-old seedling of IISR Kodagu Suvasini were used as host plants. In banana to banana combination, two banana plants were used and in banana to cardamom combination five cardamom plants were used. While, in cardamom to cardamom combination, five plants of cardamom was used and in cardamom to banana combination, four banana plants were used. The inoculated plants were maintained in the glass house and monitored for the manifestation of symptoms (if any). The plants did not develop any visible symptoms even after four months after inoculation and absence of the virus in the inoculated plants was confirmed by performing real time-PCR.

Transmission studies of BBrMV through seeds

Seed transmission studies were carried out using immature capsules, immature and mature seeds collected from virus infected plants of the cv. Njallani Green Gold. In RT-PCR, all the samples showed a clear amplification of 950 bp for BBrMV, indicating the presence of the virus in immature capsules and immature and mature seeds tested (Table 16). Grow-out test was also carried out with the seeds collected from infected capsules. The seeds were sown in separate seed pans and kept in insect proof glass house. All the seedlings were asymptomatic and the absence of both the viruses in the seedlings was confirmed by performing RT-PCR (Table 17).

Table 16: Testing seeds and capsules from infected plants for BBrMV by RT-PCR

Capsules/ seeds tested	No. of Capsules/ seeds tested		No. of Capsules/ seeds positive in RT-PCR		% of infection	
	BBrMV	Healthy	BBrMV	Healthy	BBrMV	Healthy
Immature capsules	14	7	14	0	100	0
Immature seeds	35	7	35	0	100	0
Mature seeds	35	7	35	0	100	0

Table 17: Details of seed transmission study of BBrMV						
No. of seeds sown	No. of seeds germinated	Days taken for germination	% of germination	Symptomatic seedlings	No. of seedlings tested	No. of PCR $+^{ve}$ seedlings
250 (from infected source)	200	25-28 days	80.0	0	50	0
100 (Healthy)	82	21-26 days	82.0	0	10	0

(IV) Development of Reverse Transcription- Polymerase Chain Reaction (RT-PCR) and real time- PCR based detection of BBrMV

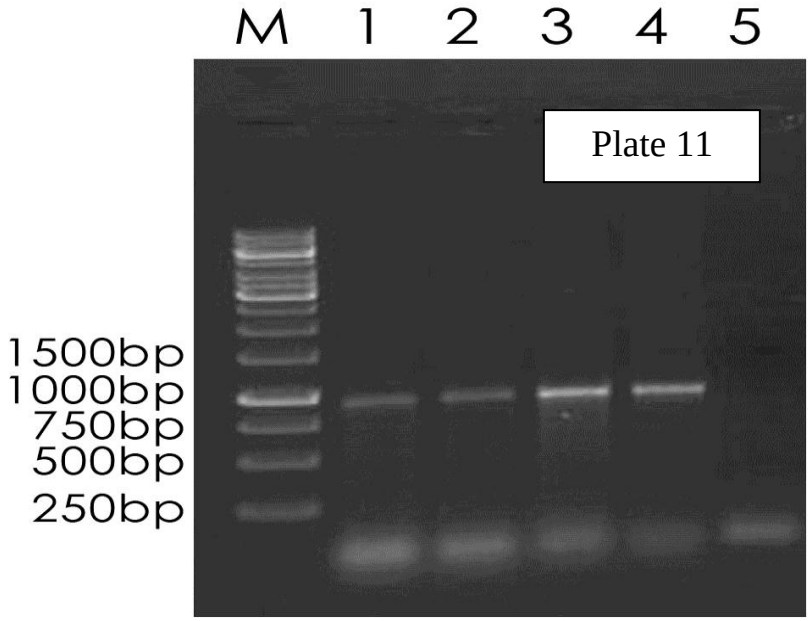
Development of Reverse Transcription- Polymerase Chain Reaction (RT-PCR) and validation

Protocols for RNA isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for detecting BBrMV in cardamom plants was developed.

The standardized protocols for RNA isolation and RT- PCR conditions are as follows:

Total RNA from 100 mg of cardamom leaf tissue was extracted by grinding infected leaf in 500 µl TRI reagent containing 0.1% ß-mercaptoethanol. To this homogenate, 50 µl of 2M sodium acetate pH 4.0, 500 µl of water saturated phenol and 100 µl of chloroform: isoamyl alcohol (49:1) was added serially with intermittent mixing at each step. The mixture was then shaken for 10 s and kept on ice for 15 min. The homogenate was centrifuged at 12,000g for 15 min at 4 ºC. To the aqueous phase, 0.1 vol of 3M sodium acetate pH 4.0 and equal volume of ice cold isopropanol were added, incubated at -80 ºC for 1h and centrifuged at 12,000g for 15 min at 4 ºC. The pellet was washed with 70% ethanol; air dried and dissolved in 50 µl HPLC grade water. The RT-PCR was performed in a 50 µl reaction mixture containing 1x PCR reaction buffer (Genei, Bangalore), 20mM DTT, 100µM dNTPs (Genei Bangalore), 15 pmol of primers (forward: 5' – GCA CAT TTG GAC AAC CCA -3' and reverse: 5' – GCA CAC ATA ATT ATA GGG AG- 3'), 10 U of RNasin (Fermentas), 50 U of MMuLV RT (Fermentas), 1.5 U Taq DNA polymerase (Chromus), 20µl template RNA and sterile water to a final volume of 50 µl. The thermal cycler was programmed for reverse transcription at 42 ºC for 45 min followed by 35 cycles of denaturation at 94 ºC for 30 sec, annealing at 54 ºC for 1 min, extension at 72 ºC for 1 min and final extension at 72

ºC for 10 min. The RT-PCR product was analyzed on 1% agarose gel. The expected size of ~950bp was observed in infected cardamom plants. No such band was seen in healthy samples (Plate 11).



Lane M: Marker (1 kb ladder); Lanes 1 -4: chlorotic streak infected cardamom samples; Lane 5: Healthy sample

The RT-PCR based method was subsequently validated by testing cardamom field samples originating from different geographical locations (Table 18).

Table 18: Detection of BBrMV from field samples of cardamom collected from different regions through RT-PCR

State	District	No. of samples tested			No. of positive in RT-PCR		
		Symptomatic	symptomless tillers (collected from infected clump)	symptomless plants (around infected clump)	Symptomatic	symptomless tillers (collected from infected clump)	symptomless plants (around infected clump)
Karnataka	Kodagu	2	0	1	2	0	0
	Hassan	3	0	1	3	0	0
	Uttara Kannada	8	2	2	8	2	0

Kerala	Wayanad	3	3	3	3	3	1
	Idukki	6	3	3	6	3	0
Total		22	8	10	22	8	1

Standardization of real time PCR based detection of BBrMV

The specificity of SYBR green assay was evaluated using three different reactions, which included infected sample, healthy (negative) control and water (without template) control. In order to check the influence of RNA extraction method, real time assay was performed with RNA extracted by manual and kit methods separately.

The real time PCR products were analyzed by agarose gel electrophoresis. The assay with BBrMV sample displayed the expected band of 117 bp, whereas healthy and water control did not amplify (Plate 12).

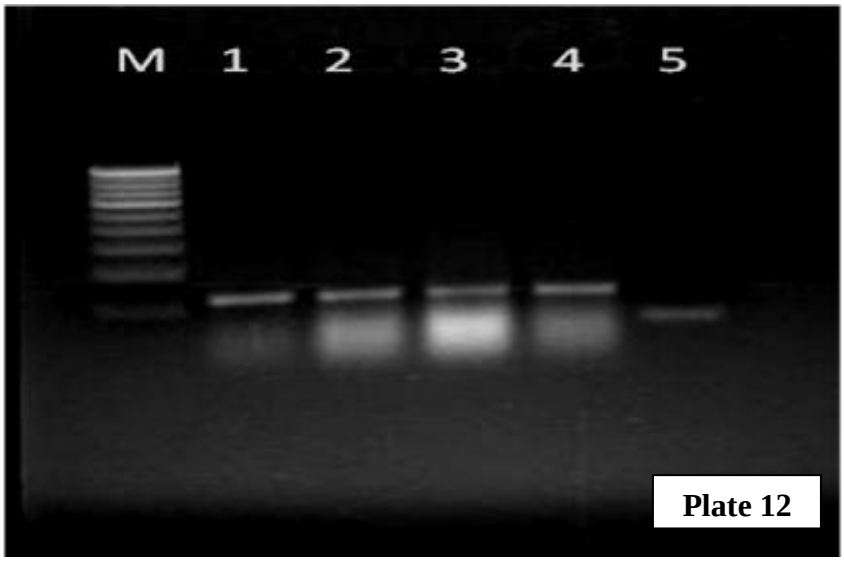


Plate 12

Lane M- 100bp ladder, Lane 1-BBrMV isolate from Appangala, Lane 2- BBrMV isolate from Idukki, Lane 3- CdMV isolate from Appangala, Lane 4- CdMV isolate from Idukki, Lane 5-Healthy control (using BBrMV specific primers).

The sensitivity of SYBR green assay was tested using different template RNA volumes *viz.* 0.01, 0.1, 1.0, 3.0 and 5.0 µl template for RNA isolated by manual method and 0.005, 0.01, 0.1, 1.0, 3.0, 5.0, 7.0 and 10.0 µl template for RNA isolated by kit method. For manual method, the SYBR green RT-PCR could detect the presence of

BBrMV in the infected sample in the range of 0.01 µl to 5 µl, with Ct values ranging from 15.78-32.12 (Table 19 and Fig. 5).

Table 19: Ct values of BBrMV detection in real time RT-PCR using the templates isolated by manual method

RNA template volume	Ct values of BBrMV
0.01 µl	31.12
0.1 µl	25.0
1.0 µl	19.23
3.0 µl	16.17
5.0 µl	15.78

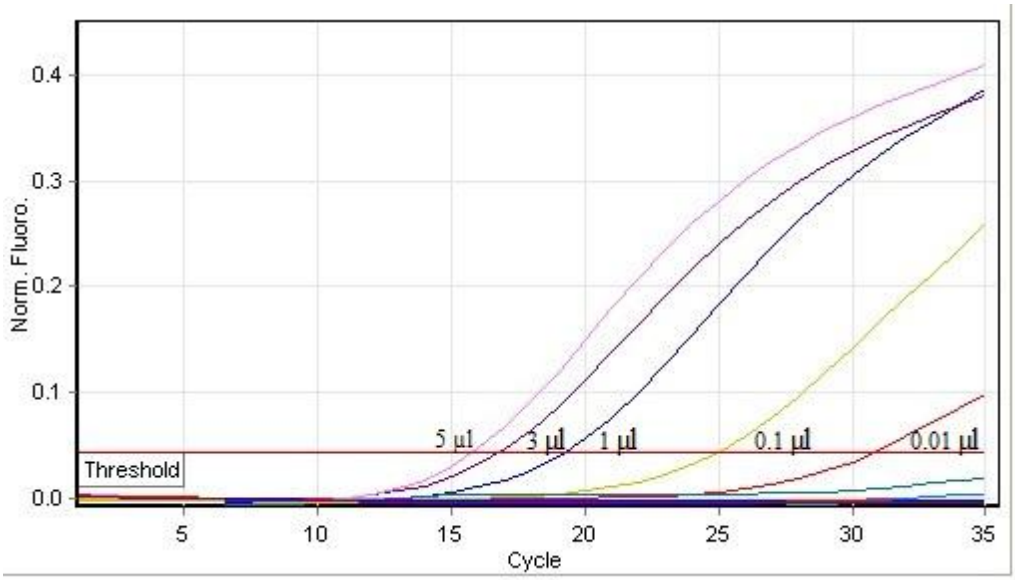


Fig. 5: Real time RT-PCR detection of BBrMV infected cardamom sample with manual method. Amplification plots show the sensitive threshold as 0.1 µl with a Ct value of 31.12. The water control and healthy control did not result any signals.

For kit method, the SYBR green RT-PCR could detect the presence of BBrMV in the infected sample in the range of 0.005 µl to 10 µl, with Ct values ranging from 14.60-27.14 (Table 20 and Fig 6). However, the real time assay with template isolated by manual method could detect the virus at a minimum concentration of 1 µl. This indicates that, the sensitivity of SYBR green RT-PCR could be increased by using template isolated by kit method.

Table 20: Ct values of BBrMV detection in real time RT-PCR using the template isolated by kit method	
RNA template volume	Ct values of BBrMV
0.005 µl	27.14
0.01 µl	25.09
0.1 µl	19.13
1.0 µl	15.11
3.0 µl	14.60
5.0 µl	15.32
7.0 µl	15.71
10.0 µl	18.34

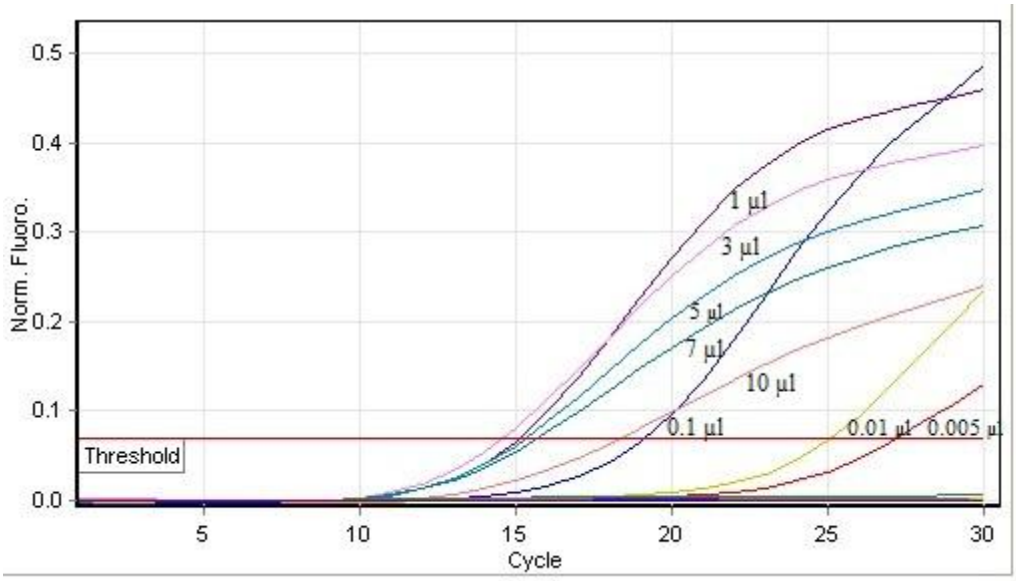


Fig. 6: Real time RT-PCR detection of BBrMV infected cardamom sample using the templates isolated by kit method. Amplification plots show the sensitive threshold as 0.005 µl with a Ct value of 27.14. The water control and healthy control did not result any signals.

Multiplex RT-PCR (mRT-PCR) based detection of *Cardamom mosaic virus* (CdMV) and *Banana bract mosaic virus* (BBrMV)

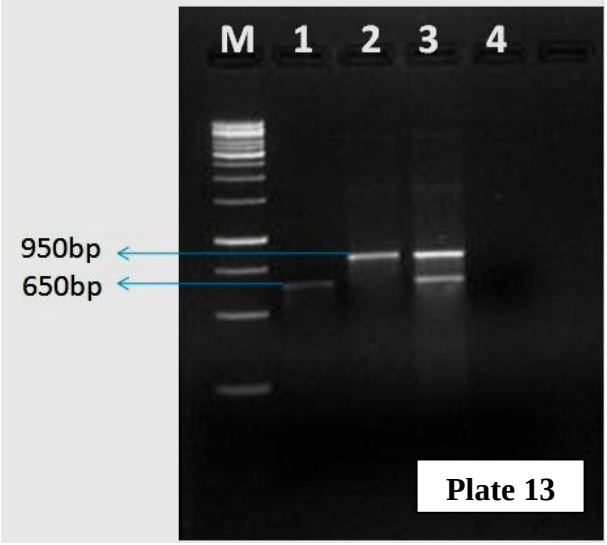
Total RNA isolated from 100 mg of leaf tissue following the method described by Siju *et al*. (2007) was used for isolation of template RNA. Two pairs of compatible virus specific primers suitable for single tube mRT-PCR were designed on the basis of coat protein sequence data of cardamom isolates of CdMV and BBrMV (Table 21). The RT-

PCR was performed in a 50 µl reaction mixture containing 1x PCR reaction buffer, 0.1M DTT, 25 mM dNTPs, 15 pmol each of the four primers, 10 U of, 50 U of RT, 1.5 U Taq DNA polymerase, template RNA and sterile water to a final volume of 50 µl. The thermal cycler was programmed for reverse transcription at 42 ºC for 45 min followed by 35 cycles of denaturation at 94 ºC for 30 sec, annealing at 56 ºC for 1 min, extension at 72 ºC for 1 min and a final extension at 72 ºC for 10 min.

Table 21: Primers used in the m RT-PCR detection of BBrMV and CdMV

Virus	Primers	Sequence	Amplicon size
CdMV	AIB 44	5'- GAAAACCCACAAAAACTCCC -3'	850 bp
	AIB 75	5'- CACCGCTTGCACCAATGAC -3'	
BBrMV	AIB 62	5'- GCACACATAATTATAGGGAG -3'	650 bp
	AIB 154	5'- AAGCCTGATCAGTTTGATTTG -3'	

The mRT-PCR amplified product was analyzed on 1% agarose gel. The results showed the presence of two bands of expected size (650 bp for BBrMV and 850 bp for CdMV). No such bands were observed in healthy plants (Plate 13).



Lane M: 500bp ladder, Lane 1: BBrMV infected sample, Lane 2: CdMV infected sample, Lane 3: Mixed infection with BBrMV and CdMV, Lane 4: Healthy (negative) control

8222. Questions answered:

(1) Is it possible to develop serological based diagnostic tools for the detection of viruses infecting small cardamom?

Yes. A protocol for purifying *Cardamom mosaic virus* from the infected leaf material was standardized and these purified virus preparations was further used for the production of polyclonal antiserum and subsequently used for the development of ELISA based detection. Of the three different dilutions of polyclonal antiserum (1:250, 1:500, 1:1000) tested, 1:250 dilution gave higher absorbance compared to the other dilutions.

(2) Is it possible to develop nucleic acid based diagnostic tools for the detection of viruses infecting small cardamom?

Yes. Protocols for RNA isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for detecting CdMV were standardized. The standardized protocol could detect the virus at 2 and 7 µl template concentrations from the infected samples. Further, the protocols developed were validated using field samples of cardamom belonging to different geographical locations and were found to be reliable. Similarly, protocols were developed for total RNA isolation and RT-PCR for detecting BBrMV was developed and validated by testing field samples of cardamom and found to be reliable.

(3) Is it possible to detect CdMV and BBrMV simultaneously in mixed infections?

Yes. The protocol developed for multiplex Reverse Transcription-Polymerase Chain Reaction (mRT-PCR) for detecting both CdMV and BBrMV could be of immense help in detecting both the viruses in mixed infections.

8223. Process/Product/Technology developed:

Process: - Standardization of real time-PCR based detection of CdMV and BBrMV.

Technology: -

(1) RT-PCR based protocols for the detection of *Cardamom mosaic virus* (CdMV) and *Banana bract mosaic virus* (BBrMV) infecting small cardamom was developed and validated using field samples.

(2) mRT-PCR (multiplex reverse transcription – polymerase chain reaction) based detection of both CdMV and BBrMV infecting cardamom.

8224. Practical utility (not more than 150 words):

In the present investigation, occurrence of a new viral disease of cardamom named as “chlorotic streak” incited by a strain of *Banana bract mosaic virus* (BBrMV) in the cardamom growing tracts of Karnataka and Kerala was revealed for the first time. Surveys conducted in 77 plantations belonging to 49 locations revealed that the disease incidence is in the range of 0 – 15%. The disease was characterized by the formation of continuous or discontinuous spindle-shaped yellow or light green intravenous streaks along the veins and midribs. Leaf dip electron microscopy showed the presence of flexuous particles resembling Potyvirus in the symptomatic samples. Subsequently, cloning and sequencing studies indicated that, the causal agent is a strain of BBrMV. A nucleic acid based (RT-PCR) protocol for the detection of the virus was developed and validated using field samples. A real-time PCR based detection method and multiplex-PCT detection protocol (for the simultaneous detection of CdMV and BBrMV) was also developed.

The nucleic acid based diagnostic procedures developed in this project for the detection of CdMV and BBrMV either singly or in mixed infections would immensely help to detect both the viruses even in asymptomatic plants. The technology which is sensitive and reliable could be used to index the mother stock, so that disease free planting material can be obtained and multiplied on a large scale to establish a disease-free plantation.

823. Publications and Materials Development:

(One copy each to be supplied with this proforma)

8231. Research Papers: (copies enclosed)

(1) C. N. Biju, A. Siljo and A. I. Bhat (2010) Survey and RT-PCR based detection of *Cardamom mosaic virus* affecting small cardamom in India. *Indian Journal of Virology*. 21: 148–150.

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

Research paper communicated:

A. Siljo, A. I. Bhat and C. N Biju. Symptomatological and coat protein gene sequence studies suggest high variability in *Cardamom mosaic virus* isolates occurring in India (*communicated to Journal of Spices and Aromatic Crops*).

8232. Popular articles: (copies enclosed)

(1) A.I. Bhat, C.N. Biju and A. Siljo. (2010) Chlorotic streak – a new virus disease of cardamom in India. *Spice India*. 23 (12): 10 – 12.

(2) Ankegowda, S. J., Venugopal, M. N., Biju, C. N. and Senthil Kumar, R. (2012) Problems and prospects of spice crops production in Kodagu. *Spice India*. 25 (3). 9 – 15.

(3) M. N. Venugopal, S. J. Ankegowda and C. N. Biju (2008) *Management of viral disease in cardamom*. Published by Spices Board, Sakleshpur (Extension pamphlet in Kannada).

A. Siljo, A. I. Bhat, C. N. Biju and M. N. Venugopal. Characterization of the virus associated with chlorotic streak, a new disease of cardamom in India.
(Paper presented *In "Symposium on Changing plant disease scenario in relation to climate change" Organized by Indian Phytopathological Society (Southern Zone) and Indian Institute of Spices Research (ICAR), Kozhikode from 22nd to 23rd October, 2010*).

Technical bulletin entitled *"Viral diseases of cardamom"* authored by C. N. Biju and A. I. Bhat is under preparation.

8233. Reports: Annual Report, IISR, Kozhikode (2008 - 2012).

8234. Seminars and workshops (relevant to the project) in which the scientists have participated:

Sl. No.	Scientists	Seminar/ Workshop	Date	Venue/Location
1.	Dr. Biju. C.N.	Regional Seminar on Pepper and Cardamom	23rd March,2009	IISR, Cardamom Research Centre, Appangala.
2.	Dr. Biju. C.N.	National Seminar on Horticulture Biotechnology	28th to 29th October, 2009	Indian Institute Horticultural Research, Bengaluru, Karnataka.
3.	Dr. A. Ishwara Bhat Dr. Biju. C.N.	National Consultative Meeting on Disease Diagnosis for Horticultural Crops	22nd to 24th, January, 2010	National Research Centre for Banana, Thiruchirapalli, Tamil Nadu.
4.	Dr. A. Ishwara Bhat Dr. Biju. C.N.	Symposium on "Changing plant disease scenario in relation to climate change"	22nd to 23rd October, 2010	Indian Institute of Spices Research, Kozhikode.

824. Infrastructural facilities developed: Nil

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

The present investigation revealed the wide spread incidence and distribution of katte disease and the existence of a new disease; chlorotic streak incited by a strain of *Banana bract mosaic virus* in the cardamom growing regions of South India. The strain of BBrMV infecting cardamom was subsequently characterized and nucleic acid based diagnostic tool was developed. Since, cardamom is cultivated under diverse cropping systems encompassing a wide variety of other crops, it is speculated that, this close association would facilitate other pathogens; especially viruses would transmigrate to cardamom apart from its natural host (s) as it was established in the case of BBrMV. Under such circumstances, this association could lead to the emergence of new diseases or could devastate the crop due to synergistic action with other viruses. Hence, explorations may be undertaken in the areas where cardamom is cultivated along with other component crops which are known hosts for other viruses. This would help in generating valuable informations regarding existence or non-existence of new diseases of cardamom. Further, investigations on the epidemiology and transmission pattern would immensely help to formulate effective management measures to contain the diseases.

Part – IV – Project Expenditure
(Summary)
2008 – 2012

830	**Total Recurring Expenditure**		
8301	**Salaries**	**Estimated**	**Actual (Rs.)**
	(i) Scientific	**7,80,000**	**11,34,000**
	(ii) Technical	**1,90,000**	**1, 60,000**
	(iii) Supporting	**54,000**	**48,000**
	(iv) Wages (Contractual)	**-**	**-**
	Sub Total	**10,24,000**	**13,42,000**
8302	**Consumables**		
	(i) Chemicals	**2,00,000**	**35,000**
	(ii) Glasswares	**40,000**	**6,000**
	(iii) Others	**40,000**	**5,000**
	Sub Total	**2,80,000**	**46,000**
8303	**Travel**	**25,000**	**13,000**
8304	**Miscellaneous (other costs)**	**-**	**6, 000**
8305	**Sub Total (Recurring)**	**13,29,000**	**14,07,000**
831	**Total Non-Recurring Expenditure (Equipments and works)**	**-**	**-**
Total (830 and 831)		**13,29,000**	**14,07,000**

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, material are available for the same.

Signature of the Project Investigator: Biju. C. N.

Signature of Co – Principal Investigator: A. Ishwara Bhat

Signature and comments of the Head of the Centre:

Signature and Comments of the Head of the Division/ Section:

Signature and Comments of the Director: