CHARACTERIZATION OF DIFFERENT VIRUSES INFECTING SMALL CARDAMOM (*Elettaria cardamomum* Maton) AND PRODUCTION OF DISEASE FREE PLANTS

by

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"Characterization of different viruses infecting small cardamom (*Elettaria cardamomum* Maton) and production of disease free plants"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associate ship, fellowship or other similar title, of any other University or Society.

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Vangala Bhavana

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LIST OF ABBREVIATIONS

2,4-D	2, 4- dichlorophenoxy acetic acid
µg	Microgram
µg/ml	Microgram per millilitre
µl	Microlitre
A	Adenine
AgMV	*Agropyron mosaic virus*
AlpMV	*Alpinia mosaic virus*
bp	Base pair
BA	Benzyl adenine
BAP	Benzyl amino purine
BBrMV	*Banana bract mosaic virus*
BCIP	Bromo chloro indolyl phosphate
BLAST	Basic local alignment search tool
BLASTn	Basic local alignment search tool
BSA	Bovine serum albumin
C	Cytosine
Ca	Calcium
et al.	Co-workers
cDNA	Complementary DNA
CdMV	*Cardamommosaic virus*
ChYMV	*Chinese yam mosaic virus*
Cm	Centimeter
CVYV	*Cucumber vein yellowing virus*
CP	Coat protein
DAC-ELISA	Double antibody coating- Enzyme linked immunosorbent assay
DAS-ELISA	Double antibody sandwich- Enzyme linked immunosorbent assay
FP	Forward Primer
g	gram
ha	Hectare
IAA	Indole Acetic acid

IBA	Indole Butyric acid
M	Molar
MEGA	Molecular evolutionary genetics analysis
mgL^{-1}	milligram per liter
mm	Millimeter
NCBI	National Center for Biotechnology Information
OD	Optical density
pNPP	Para nitro phenyl phosphate
PVY	*Potato virus* Y
RNA	Ribonucleic acid
rpm	Revolution per minute
RT-PCR sec	Reverse transcription-polymerase chain reaction Second
SDM	Spray dried milk
Spp.	Species
SPMMV	*Sweet potato mild mottle virus*
T	Thymine
TAE	Tris-acetate EDTA buffer
TBS	Tris buffer saline
TBS-SDM	Tris buffer saline-Spray dried milk
viz.	namely
@	At the rate of
%	Percent
°C	Degree Celsius

Introduction

1. INTRODUCTION

Small cardamom is one of the oldest spices in the ancient world. It is also known as the queen of spices. The name *Elettaria cardamomum* has been derived from Tamil language (Abu-Taweel, 2018). It is the third most expensive crop after vanilla and saffron. It is the second highest exported spice crop after pepper. It grows as a native of South Indian Western Ghats. The crop is sensitive to wind, rain, water-logging and drought. It grows in the forest areas with loam soils rich in potassium, medium in level with phosphorous and a pH range of 5.5 – 5.6 (Ankegowda *et al.*, 2015) within shade and damp areas. It grows well in the temperature of 10-35^{0}C with average rainfall of 1500 mm and slope of 600-1500 m (Reyes *et al.*, 2006).

In India, cardamom is cultivated in Kerala, Karnataka and Tamil Nadu. In Kerala the crop is mostly cultivated in Wayanad and Idukki districts. In Karnataka, Uttara Kannada, Shivamogga, Hassan Chickmagalur, and hills of Kodagu (Coorg) districts are the major cardamom cultivated areas. Northern and Southern foot hills of Nilgiris, Madurai, Salem and Tirunelveli, Annamalai and parts of Coimbatore districts of Tamil Nadu are the major cardamom growing tracts (Madhusoodanan *et al.*, 1994).

Kerala being called as "Land of Spices" and the spices export from Kerala during 2017-2018 was 1, 40,050 with an area of 1, 62,660 ha. In Kerala small cardamom is cultivated in about 39,080 ha with a production of 18,350 tonnes (Spice Board, 2017-2018).

Small cardamom is infected by fungal, bacterial and viral diseases. Damping off, and clump rot and fruit rot are the major problem especially in shady area (Phadnis, 2015). The major constraints in cardamom production are due to the viral diseases. Small cardamom is majorly found to be affected by four viral diseases that cause significant yield loss. The viral diseases of small cardamom include katte disease, chlorotic streak disease, cardamom necrosis disease, and cardamom vein clearing disease. Out of the four viral diseases, katte disease and chlorotic streak disease were found to be prominent in the areas of Idukki region of Kerala as 60 per cent incidence was recorded, followed by Tamil Nadu and Karnataka.

The small cardamom plants infected with the viral diseases during pre-bearing stage show maximum yield loss (Samraj, 1970).Naidu (1983) reported that there is a mean decline in yield of about 38, 62 and 68 percent in katte infected plants. Cardamom is grown under monocropping system. So, the katte infection causes a yield loss for about 69 per cent after three years of infection, and a complete yield loss occurs after 3-5 years of infection (Venugopal, 1999). Cardamom plants infected with katte disease had shown a yield reduction of 70-100% within three years of infection (Jacob and Usha, 2001). Yield loss were noticed due to the katte infection and was up to 70 per cent, and that of cardamom vein clearing disease was upto 62-84 per cent as reported by (Ankegowda *et al.,* 2015)

The most widely followed method of propagation of the crop is by planting rhizomes with active buds. Seedling propagation is also followed but farmers prefer clonal propagation to gel consistent yield and quality capsules. The major drawbacks of vegetative propagation were the spread of diseases, especially viral diseases. To avoid this, meristem culture can be adopted which eliminates virus, and has been identified as eco-friendly and sustainable management strategy of the disease (Purseglove, 1975; Reghunath and Bajaj, 1990).

Even though there are reports of presence of viral diseases infecting small cardamom, no data is available on the distribution of viruses in major cardamom cultivation areas of Idukki district. Hence this study was undertaken with the objectives to

1) Survey for the viruses infecting small cardamom in Idukki
2) Characterization of viruses infecting small cardamom
3) Elimination of viruses through meristem tissue culture

Review of Literature

2. REVIEW OF LITERATURE

Small cardamom (*Elettaria cardamomum* Maton) is grown as one of the exports earning spice crop cultivated in areas of hilly elevations in Kerala. The crop is affected by viral diseases like katte disease and chlorotic streak disease

The outline of technical programme includes

1) Survey
2) Symptomatology
3) Serological diagnosis and Molecular characterization of the viruses.
4) Elimination of virus through meristem culture.

2.1 SURVEY

In South India, 'Katte' is widely distributed in all the cardamom growing tracts with incidence ranging from 0.01 to 99.00 per cent (Mayne, 1951). A systematic survey conducted during 1980-81, revealed that the disease was prevalent in most of the cardamom growing districts of Kerala, Karnataka and Tamil Nadu except Trivandrum, Kanyakumari, Tirunelveli and Shimoga. Varma and Capoor (1953) and Deshpande *et al.*, (1972) found that katte disease caused low yield and rapid decrease in cardamom plantations. The disease incidence varied from 0.01 to 99 per cent in different plantations (Naidu *et al.,* 1984).

Venugopal (1995) reported that the cardamom growing regions of Tamil Nadu had cardamom necrosis disease incidence ranging from 7.7 to 80.0 per cent (Sridhar *et al.,* 1990) reported that in Kerala, in some estates in Munnar and Thondimalai areas of Idukki, cardamom necrosis disease incidence of 4.6 and 1.46 per cent respectively was recorded.

Ninety eight plantations in North Kanara, Hassan and Kodagu districts in Karnataka state were surveyed and 5 representative plantations were selected for disease management trials. Rouging of the infected plants in all the 5 plantations was found very effective to contain further spread of both 'Katte' and 'Kokke kandu' diseases.A survey of six plantations in Hongadahalla revealed an incidence of katte ranging from 10.4 - 42.8 per cent in different plantations (Venugopal, 1995; Venugopal, 2002).

Jacob and Usha (2001) reported that there was 70-100 per cent reduction in yield of cardamom due to katte disease caused by Cardamom mosaic virus (CdMV) within three to four years. The infected plants were stunted and remained to be the source of virus inoculums to other healthy cardamom plants.

A survey was conducted in the cardamom-growing area covering 402 plantations in 120 villages distributed in 20 districts spanning 6,715 hectares in south India. In each plantation, 100 plants were selected for assessing the katte disease. Sixty isolates depicting the mosaic symptoms, ranging from mild to severe mosaic, were collected and maintained in the glass house (Jacob *et al.,* 2003).

Biju *et al.,* (2010) surveyed 84 cardamom plantations belonging to 44 geographical locations to study the incidence and distribution of *Cardamom mosaic virus* in Kerala and Karnataka during 2008–2009, and found that the katte disease incidence ranged from 0 – 12 per cent in Idukki region and 0-85 per cent in Karnataka region.

Siljo *et al.,* (2012) and Biju (2012) reported that during the survey for cardamom mosaic or katte disease caused by *Cardamom mosaic virus* (CdMV) in cardamom plantations, a new kind of viral disease showing chlorotic streak on veins was observed in some plantations. Based on the type of symptom, the disease was named 'chlorotic streak'. The survey of 77 cardamom plantations at 49 locations in the states of Kerala, Karnataka and Tamil Nadu in India, Siljo *et al.,* (2012) and Biju (2012) reported chlorotic streak disease incidence ranging from 0 to 15 per cent in the plantations where either banana was grown nearby or banana was the previous crop. The highest disease incidence was recorded from Vythiri taluk of Wayanad district in Kerala.

2.2 SYMPTOMATOLOGY

Cardamom mosaic virus (CdMV) is the causative agent of katte or cardamom mosaic, the most widespread and devastating disease of small cardamom. It was locally known as "marble disease", and first reported by Buchanan in 1807 from India. Bundren (1914) reported that the disease infected plants becomes unproductive after 3-4 years of

infection. The virus was transmitted by the banana aphid *Pentalonia nigronervosa* (Uppal *et al.*,1945; Varma, 1962).

The first visible symptom was found on younger tiller as a slender chlorotic fleck, later develops into pale green discontinuous stripes. These lines run parallel from midvein region to leaf margin. Sometimes mosaic type mottling could be seen on the pseudostems and leaf sheaths. In the advanced stage of infection, the plants produced shorter and slender tillers. Capoor (1967) reported that the plant infected with katte disease had shown no malformation, but the size of leaf and pseudostem were decreased.

Rao and Naidu (1973) observed symptoms like stunted growth and mosaic symptoms on the pseudostems of the katte infected plants, whereas the symptoms on the mature pseudostems were not seen as in the younger plants. Nambiar *et al.,* (1975) reported that the disease infection at seedling stage resulted in the failure of flowering and fruit bearing capacity. Rao (1977) classified the katte infected samples into three types and categorized as A, B and C isolates based on variations in symptoms produced. Isolate A had shown dark longitudinal stripes, B isolate had shown broken dark green bands with chlorotic stripes and C isolate had shown stunted growth with granular mosaic pattern on the leaf portion.

Dimitman *et al.,* (1984) reported the presence of new shoots with mild chlorosis with greenish yellow mottling and reduced growth rate in katte disease affected plants. Gonsalves *et al.,* (1986) reported the symptoms of dark green stripes running on the light green background, which resulted a distinct mosaic pattern in katte disease affected leaves and petiole. In case of severe infection, the plants die in 4-5 years after the appearance of symptoms. Siddharamaiah *et al.*, (1991) reported 50 per cent loss of fresh weight of capsules in the katte affected cardamom plants.

Venugopal (1995) reported that the characteristic symptoms of katte disease was the presence of stripes of green tissue evenly distributed over the leaf lamina and mosaic type mottling on leaf sheaths and young pseudostems. In the case of severe infection, the affected plants produced stunted tillers.

Biju *et al.,* (2010) reported that viral diseases in small cardamom cause serious economic loss to the farmers growing the crop in regions of Karnataka and Kerala. The

disease is caused by Cardamom mosaic virus(CdMV) belonging to the genus *Macluravirus* in the family *Potyviridae.* Under monocrop conditions,the decline of the crop occurred in a span of 3–4 years of infection. The disease is characterized by prominent discontinuous yellowish stripes running out from midrib to the margin of young leaves. Primary spread of the disease is through infected clones while secondary spread occurs through non-persistent transmission by the aphid, *Pentalonia nigronervosa f. sp. caladii.*

Siljo *et al.,* (2012) reported that in Karnataka, the small cardamom varieties SKP-14 and CCS-1 were grown on a large scale, while in Kerala the popular cultivated variety was Njallani Green Gold. The katte disease caused by *Cardamom mosaic virus,* produced similar symptoms on all the cultivated varieties grown in Kerala and Karnataka with some small variations. In Karnataka isolates, light green with prominent chlorotic streaks were found while in the case of Kerala isolates light/dark green mosaic patches were seen along the midvein region. Other symptoms like mottling of leaf sheath and pseudo-stem, and short slender tillers were common in both.

Bhat *et al.,* (2018) reported that the symptoms of katte disease vary with different virus isolates pertaining to different geographical locations. Severe infection led to decreased tiller production. The virus was primarily transmitted by banana aphid *Pentalonia nigronervosa* Coq. and secondarily by infected clumps.

Siljo *et al.,* (2012) reported the occurrence of chlorotic streak disease caused by *Banana bract mosaic virus* (BBrMV) in small cardamom for the first time. On the infected small cardamom plants, the symptoms commonly seen were continuous or discontinuous spindle shaped intravenous streaks along midrib and midvein. These streaks later coalesce with each other and imparted yellow or light green colour to the veins. Discontinuous spindle shaped mottling was seen on petioles and pseudo stem. In severe cases, tillers will be suppressed. The most distinguishing feature was the formation of intravenous streaks due to chlorosis, hence the disease name was referred to as 'chlorotic streak'.

Tiwari *et al.,* (2016) reported that *Cardamom mosaic virus* (CdMV), belongs to the genus Macluravirus of family Potyviridae. In diseased plants, the symptoms included interrupted pale green stripes which ran along the veins and parallel to each

other from midrib to margin of the leaf. In advanced stages, the pale green stripes were distributed evenly over the leaf surface giving a distinct mosaic pattern. Bhat *et al.*, (2018) reported that in small cardamom variety Njallani Green Gold symptoms like intravenous streaks, mottling of pseudo stem and petiole, loosening of leaf sheath and stunting of the plants were expressed as a result of BBrMV infection.

2.3 SEROLOGICAL DIAGNOSIS AND MOLECULAR CHARACTERIZATION OF THE VIRUSES INFECTING ABOVE CROPS

2.3.1 DAC-ELISA - Direct antigen coated-enzyme linked immunosorbent assay

The most common serological technique used for the detection of plant viruses is ELISA, which is based on antiserum raised against virus specific proteins. This serological technique is being routinely used for the detection of plant viruses based on the principle of antigen – antibody reaction (Voller *et al.*,1976;Clark and Adams, 1977; Gould and Symons, 1983). It has been found to be a sensitive method and could be used to detect viruses in previously intractable tissues such as the flesh of potato tubers, leaves of tree, fruits and the bulbs of bulbous ornamentals (Torrance and Johnes, 1981; Cooper and Edwards, 1986).The sensitivity of ELISA varies according to the sample's freshness, virus titre and the organism/tissue infecting. Both monoclonal and polyclonal antisera could be used, where only single epitope is recognised by monoclonal antisera which are more expensive whereas multiple sites are recognised by polyclonal antisera and are less expensive (Martinelli *et al.*, 2015)

Potyvirus is the largest genus group that contains about 91 formal species and 88 tentative species. These cause significant losses in various plants with the aphid transmission. Since 1970's the ELISA test is being used widely for the detection of viruses. Koenig (1981) has reported that potyviruses could be detected by using direct and indirect ELIA. Hobbs *et al.*, (1987) standardised the protocol for DAC-ELISA for detecting the viruses infecting peanut. DAC-ELISA was more preferred when compared to other methods.

Jacob *et al.*, (2003) reported strain variations in isolates of CdMV from different geographical regions of Karnataka and Kerala based on their reaction to the antiserum developed against Yeslur isolate of CdMV. Smitha (2004) performed DAC-ELISA by raising polyclonal antisera. DAC-ELISA was performed by using the antigen

extracted in carbonate buffer at different dilutions of (1:10, 1:100 and 1:1000) and different antisera dilutions (1:100, 1 :500, 1:1000, I :2000, 1 :4000, 1:6000 and 1:10000). The virus CdMV was detected at 1:1000 antigen dilution with 1:4000 and 1:10000 antisera dilutions. The serological relationship between CdMV with other virus groups using DAC-ELISA was also studied.

In a study conducted by Biju (2012), ELISA was used for the detection of *Cardamom mosaic virus* using three different dilutions of antisera 1: 250, 1: 500 and 1: 1000. Alkaline phosphatase labelled goat anti rabbit antibody was used at the dilution of 1:7500. Of the three dilutions of antisera used, the dilution 1:250 recorded higher absorbance for the CdMV isolates collected from different regions of Kerala, Karnataka and Tamil Nadu.

Phadnis (2015) conducted both direct and indirect ELISA for the detection of CdMV. In the case of direct ELISA, primary antibody in the dilution ratio of 1:500 and 1:1000 in combination with antigen dilutions like 1:1, 1:10, 1:50, 1:100 were used. The yellow colour was obtained with the reaction of secondary antibody at 1:1000 dilution. In the case of indirect ELISA, the antigen and antibody concentrations varied with different isolates. Sakleshpur isolate produced yellow colour with OD value of 1.023 and 1.061, when the antigen was diluted at 1:100, 1:1000 respectively and secondary antibody dilution was 1:1000. The sample from Mudigere recorded OD value of 1.072 and 0.976 with the antigen dilutions of 1:100 and 1:50 respectively and with primary and secondary antibody dilutions of 1:1000.

Arya (2016) carried out indirect ELISA for the confirmation of katte disease of small cardamom. The antibodies obtained from the rabbit blood samples were used as antibody sources. Primary antibody was used at the ratio of 1:10, 1:100, 1:150 and secondary antibody at the ratio of 1:200. The result of indirect ELISA revealed that the crude sap of infected cardamom leaves cross reacted with the antibody specific for *Banana bract mosaic virus* (BBrMV) which also belongs to same potyvirus group.

Tiwari *et al.,* (2016) demonstrated that indirect ELISA was standardized using different dilutions of infected plant sap or ultra-purified virus as antigen. The primary antibody dilutions used were 1:500, 1:1000, 1:2000 and secondary antibody dilutions

were 1:1000 and 1:2000. The antigen dilutions used were 1:10, 1:50, 1:100, 1:200, 1:500 and 1:1000. The virus could be detected at 1:1000 dilution of primary antibody and 1:2000 dilution of secondary antibody for indirect ELISA. In case of direct ELISA, CdMV could be detected at 1:400 primary antibody dilution and 1: 800 for secondary antibody dilution.

2.3.2 DIBA-Dot immunobinding assay

Dot immunobinding assay is a simple diagnostic technique where virus is directly bound to the nitrocellulose membrane and is detected with virus specific antibodies that are in turn detected with antibody enzyme conjugates. It is also referred as Dot-ELISA. It is a simple diagnostic technique, which is less expensive and labour intensive than conventional ELISA procedures and has been used to detect a variety of plant viruses.

Smitha (2004) used DIBA to detect the presence of CdMV at a dilution of 1:10, 1:100 and 1:1000. As this method was sensitive, it gave the positive results all the infected samples even at the dilutions of 1:1000 with blue colour development on nitro cellulose membrane. DIBA was carried out by Phadnis (2015), by raising the antibodies in rabbit. The antigen dilutions used as a titre fixation was 1:100. The antisera used in the combinations of 1:500, 1:1000 and 1:2000 respectively with secondary antibody dilution of 1:1000. In all the combinations purple colour development was seen, optimum reaction was observed in the 1:100 antigen, 1:1000 primary antibody and 1:1000 secondary antibody dilutions.

Tiwari *et al.,* (2016) performed Dot-Blot on nitrocellulose membrane which was dipped in boiling water and equilibrated in PBS. Two microlitre of 1:10, 1:100 and 1:500 dilution of ultra-purified virus was loaded on the membrane using the impression of wells from a manifold with a control, which was the buffer. The primary antibody dilutions used were 1:500 and 1:1000 while that of secondary antibody was 1:1000.

2.3.3 Electron microscopy

Naidu *et al.,* (1981; 1984) studied the morphology of CdMV by electron microscopy and observed that the virus were flexuous filamentous particles with 650nm in length and 11-12 nm in breadth. Dimitman*et al.,* (1984) revealed the structure of CdMV as flexuous filamentous particles showing helix symmetry. Gonsalves *et al.,*

(1986) revealed the shape of CdMV as flexuous filamentous particles in the infected leaf samples with the help of electron microscopy.

Jacob and Usha (2001) reported that CdMV causing katte in small cardamom was filamentous flexuous particles in electron microscopy using 2 per cent uranyl acetate and lead acetate. The virus was isolated by density gradient ultracentrifugation, and the presence of filamentous particles with ~ 800 nm lengths and 11-12 nm breadth were confirmed. Smitha (2004) reported that electron micrograph of CdMV indicated the presence of numerous flexuous shaped filamentous particles with a size of 650-710nm in length and 12-13nm in width. Tiwari *et al.*, (2016) reported that electron microscopy of infected samples with 2 per cent uranyl acetate had shown flexuous rod shaped particles that were aggregated numerously with 700nm length.

2.3.4 Molecular diagnosis

Different viruses produce similar virus symptoms and in order to detect the particular virus causing infection, molecular test is a compulsory tool (Webster *et al.*, 2004).Polymerase chain reaction (PCR) has been reported as a powerful, sensitive and versatile technique which is increasingly being used for plant virus detection. For detection of viruses with RNA genome, a modification of this technique called Reverse Transcription - Polymerase Chain Reaction (RT-PCR) has been developed (Makkouk and Kumari, 2006). It has proved to be a valuable diagnostic tool to identify RNA viruses from nucleic acid extracts of infected host plants.

Gillaspie *et al.*, (2001) detected *Cowpea aphid borne mosaic virus* (CABMV) belonging to potyvirus using RT-PCR method. The forward primer '5-CGCTCAAACCCATTGTAGAA3' and reverse primer 5'-TATTGCTTCCCT TGCTCTTTC-3' were used. An expected product of 221 bp was obtained. Jacob and Usha (2001) isolated RNA and the RT-PCR product was obtained at 1.8 kb for the amplification of Nlb and 3' UTR regions by using Poty+1 and Poty+2 primers. Smitha (2004) had performed RT-PCR using Qiagen RNeasy Kitfor amplification of two conserved regions of Nlb and 3' UTR of potyviruses with primers OligodT and MKPoty which produced an amplicon of 1.8kb.

Biju *et al.,* (2010) developed specific primers targeting the conserved region of coat protein for the detection of CdMV infected samples of small cardamom collected from Karnataka and Kerala. Siljo *et al.,* (2014) used the technique of RT-PCR and RT-qPCR for synthesis of cDNA and quantifying the amplifications by using the SYBR fluorescent dyes. They collected the samples showing symptoms of katte from Wayanad and Idukki districts, and amplified the coat protein portion of CdMV.

Phadnis (2015) isolated RNA from the CdMV infected samples of Mudigere and Sakleshpur area using lithium chloride. cDNA was synthesised by using the forward and reverse primers. 773 bp size amplicon was gel eluted, purified and used for further cloning and expression studies.

Arya (2016) detected CdMV through RT-PCR for which the total RNA was isolated from the infected and healthy plants using Trizol and converted to cDNA. A total of 11 primers were designed for the amplification of coat protein gene of the virus using Primer 3 software. The primers designed were used for synthesis of second strand of cDNA and presence of virus was detected. These amplicons were further eluted, re amplified and sequenced. The primers such as CP-1, CP-2, CP-4, CP-5 and CP-7 were shown amplifying the coat protein gene. Bhat *et al.,* (2018) reported that the whole genome sequence of BBrMV infecting cardamom showed 96.70 per cent identity to with BBrMV infected banana from Trichy and only 94.5 per cent identity to flowering ginger *Alpinia purpurea.*

2.3.3.1. *Sequencing and BLAST analysis*

The Molecular Evolutionary Genetics Analysis (MEGA) software is developed for comparative analyses of DNA and protein sequences that are aimed at inferring the molecular evolutionary patterns of genes, genomes, and species over time (Kumar *et al.,* 1994; Tamura *et al.,* 2011).

Jacob and Usha (2001) reported that CdMV isolate has shown 58.20 per cent identity with *Narcissus latent virus*(NLV)and 55.40 per cent identity with *Maclura mosaic virus* (MacMV) on constructing a phylogeny tree. It was also concluded that CdMV isolates were related to *Macluravirus.*

Jacob *et al.*,(2003) constructed a phylogeny tree where CdMV isolated from small cardamom from Sirsi in Karnataka clustered together with Vandiperiyar, Kurusupara and Kattappana isolates from Kerala state.

The phylogeny tree for CdMV isolates from Kerala and Karnataka showed 87.1- 98.9 percent identity for all Kerala isolates which were clustered together with Tamil Nadu isolates. But Karnataka isolates were clustered as one group. Six isolates infected with BBrMV in banana from India were related with BBrMV infected cardamom samples with 97-99percentidentity. Whereas an identity of 94 – 96.8percent was observed between isolates of BBrMV from Philippines, Thailand, Vietnam, Western Somalia and two other Indian isolates. Based on CP gene, 60 percent identity with *Sugarcane mosaic virus* was reported by Bhat *et al.,* (2012).

The nucleotide sequences of CdMV- CP isolates of Mudigere and Sakleshpur were studied. In the case of Mudigere isolate, CP nucleotide sequence identity was 96.50percent with *Sugarcane mosaic virus* (SCMV) (Potyvirus), 90.78percent with *Potato virus* Y (PVY) (Potyvirus), 60.36percent with *Alpinia mosaic virus* (AlpMV) (Macluravirus), 54.65percent with *Chinese yam mosaic virus* (ChYMV) (Macluravirus), 45.58percent with *Narcissus latent virus* (NLV) (Macluravirus) and least with *Onion yellow dwarf virus* (OYDV) (Potyvirus) 21.93percent. The amino acid CP sequence identity of 97.41percent with SCMV (Potyvirus), 91.88percent with OYDV (Potyvirus), 75.57percent with AlpMV (Macluravirus), 56.08 percent with ChYMV (Macluravirus), 48.33 percent with NLV (Macluravirus) and least with *Papaya ring spot virus* (PRSV) (Potyvirus).

The CP nucleotide sequence identity of Sakleshpur isolate (SKP) showed 54.51percent with *Cucumber vein yellowing virus* (CVYV) (Ipomovirus), 49.59percent with *Cardamom mosaic virus* (CdMV) (Macluravirus) and least with *Agropyron mosaic virus* (AgMV) (Rymovirus) 23.56percent. The identity of amino acid sequence of CP was 63.39percent with *Sweet potato mild mottle virus* (SPMMV) (Ipomovirus), 59.77percent with CVYV (Ipomovirus), 48.26percent with CdMV (Macluravirus) and least of 15.01percent with AgMV (Rymovirus) (Phadnis, 2015).

2.4 ELIMINATION OF VIRUSES THROUGH MERISTEM CULTURE

Plant tissue culture is the technique of *invitro* establishment of explant, a plant part like stem, root and leaf into a complete plant under a suitable medium and conditions (Adlak *et al.,* 2019). Meristem culture is one of the tissue culture techniques for the production of disease free plants. The shoot apex is selected for the propagation. The infected mother plants were selected and the shoot apex was cut at a size of 1-1.5mm size and placed into the MS medium supplemented with hormones responsible for shoot and root proliferations. This method was first adapted for the production of viral free potatoes and dahlias (Morel and Martin, 1954).

Srinivasa *et al.,* (1982) reported that the seeds of small cardamom were raised as plantlets with MS medium supplemented with 2 per cent sucrose, 2, 4-,D($2mgL^{-1}$)or indole-3-butyrie acid (IBA $2mgL^{-1}$) of naphthalene acetic acid (NAA $2mgL^{-1}$) and benzyl aminopurine (BAP $2mgL^{-1}$). Nadagudaet *al.,* (1983) had initiated the cardamom tissue culture from the young buds, with different hormones like BAP, NAA, kinetin, IAA and other additives like coconut water(5%), calcium pantothenate ($0.1\ mgL^{-1}$) and biotin ($0.1\ mgL^{-1}$).

The stored plantlets were transferred to multiplication medium (MS + 1.0 mg L^{-1} NAA) for growth stimulation before transplanting to soil. Later the plantlets reached normal size of (6-8 cm long) and had good rooting system. The shoot primordia turned green and developed into shoots after 10 days of inoculation when supplemented with $0.5\ mgL^{-1}$ kinetin. At this stage, the cultures were transferred to a MS basal medium containing $1mgL^{-1}$ of NAA. This resulted in the production of multiple shoots (1:8) and good rooting. Within 2-3 months, a good number of uniform sized plantlets were obtained (Babu *et al.,* 1990).

Babu *et al.,* (1997) reported that for shoot tip culture, MS medium was prepared by adding 0.5 per cent benzyl adenine and 1 per cent naphthalene acetic acid to multiply the roots and shoots. *In vitro* raised plantlets were established in soil with 80 per cent success after hardening in a humid chamber for 30 days, in a mixture of garden soil, sand and vermiculite in equal proportions.

Rao *et al.,* (1999) reported meristem culture of cardamom where younger rhizome explant was taken and washed under running tap water. A size of 2-3 cm size of cone

shape was obtained by trimming the outer layers. Surface sterilization was done with mercuric chloride and other antibacterial like gentamycin and tetracycline. The most appropriate explant size for culture initiation was 2-3 cm height and 1.3 – 1.6 diameter at base. The explants were inoculated on to the MS medium supplemented with coconut water. Once the primary established cultures are obtained, they are transferred to second medium containing MS+ BAP 0.5 mgL^{-1},kinetin 0.5 mgL^{-1}, IAA2 mgL^{-1}, Coconut water (5% v/v), D- biotin 0.1 mgL^{-1}andcalcium pantothenate 0.1mgL^{-1}.

The multiplication of propagules was easily accomplished by sub dividing the shoot of bud clusters and reculturing them on fresh multiplication medium. The rate of multiplication ranged from 1:1 to 1:13 with an average of 1:3 per month and the cultures were normally subcultuerd at an interval of 27- 35 days. At this stage, due to higher levels of cytokinin, rooting was inhibited in the medium, hence auxins like IBA, NAA, and IAA were tested. The plants with rooting of 4-5 cm length were hardened.

Peter *et al.,* (2001) produced somaclonal hybrids by inoculating 2-3 cm sized rhizome buds into MS medium supplemented with 0.5mgL^{-1} Kinetin. The establishment of somaclones occurred within 25-30 days. For shoot multiplication MS medium was supplemented with 1.0 mgL^{-1} BA and 0.5 mgL^{-1} NAA and resulted in maximum multiplication of shoots at 1:3 ratio. This was later transferred to MS medium supplemented with 3 per cent sucrose, and then to MS medium supplemented with O.5mgL^{-1}NAA for rooting and then finally hardened.

Smitha (2004) reported virus elimination in CdMV infected plant samples by meristem tip culture. Hormones like cytokinins were dissolved in 0.1 N HCl and auxins in 0.1 N NaOH to prepare stock solutions up to 100 ml by using double distilled water. The MS medium is added with several hormones like Kinetin 0.2mgL^{-1}+ BAP 1.0 mgL^{-1} + IBA 0.2mgL^{-1}+ NAA 0.5mgL^{-1}, coconut water 5 per cent. Ribavirin was also added in the medium to enhance the chemotherapy. pH was maintained at 5.6 to 5.8. Within two months, the multiple shoots were obtained and these were subcultured into fresh medium for further multiplication. Later the plants were subjected to virus indexing by isolating RNA using RNeasy Plant Mini Kit procured from QIAGEN, GmbH, Hilden, Germany. It was reported that all the raised plants were negative for the presence of virus in RT- PCR.

Tyagi *et al.,* (2009) standardised a protocol for slow growth conservation of cardamom by inoculating shoot tips into ½ MS media supplemented with5 mgL^{-1} BAP, 3 per cent sugar and0.7 per cent agar to obtain the multiple shoots. The cultures were obtained within 18 months and maintained at 25±2°C.Suminar*et al.,* (2019) performed meristem tip culture in Curcuma a member of Zingiberaceae and it was proved that cytokinins when added to the basal MS media, enhanced the shoot formation but inhibited the root elongation. TDZ was the best cytokinin for shoot formation.

Materials and Methods

3. MATERIAL AND METHODS

The present research work entitled'Characterization of different viruses infecting small cardamom (*Elettaria cardamomum* Maton) and production of disease free plants' was done at the Department of Plant Pathology, College of Agriculture, Vellayani during 2017-2019, with the objective to study the occurrence and distribution of viruses infecting small cardamom in Idukki district, molecular characterization of the viruses and elimination of viruses through meristem culture for the production of disease free planting material.

3.1 SURVEY AND COLLECTION OF VIRUS INFECTED CARDAMOM FROM IDUKKI

3.1.1 Survey

The survey for viral disease in cardamom was conducted in two major small cardamom growing areas of Idukki district (Plate 1), Kattappana and Nedumkandam during 2017-2019 year. Six panchayats each from two blocks Kattappana and Nedumkandam were selected (Plate 2) and 60cardamom fields / plantations were observed for the incidence of viral diseases.

3.1.1. Percent disease incidence

Percent disease incidence was calculated as

$$\text{Disease incidence} = \frac{\text{Number of infected plants}}{\text{Total number of plants}} \times 100$$

3.1.2 Maintenance of virus inoculum

Njallani or Green gold is the most commonly cultivated variety in Idukki. The healthy and diseased plants were maintained in insect proof green house in the Cardamom Research Station, Pampadumpara.

Plate 1. Map of Kerala showing Idukki district selected for conducting viral diseases survey in small cardamom

Nedumkandam	Kattappana
Thukkupalam	Pampadumpara
Chembalam	Ambalapara

Plate 2. Panchayats selected for the survey and collection of small cardamom samples infected by viral diseases in Idukki district

3.2. IMMUNO MOLECULAR CHARACTERIZATION OF THE VIRUSES

3.2.1. Enzyme linked immunosorbent assay

3.2.1.1 DAC-ELISA – Direct antigen coating- Enzyme linked immunosorbent assay

ELISA is one of the routine serological methods for the detection of plant viruses. The procedure described by Huguenot *et al.* (1993) was followed for the detection.

One gram of the infected leaf of small cardamom was homogenized in 5 ml of coating buffer (carbonate buffer) that contains 2 per cent (w/v) polyvinyl pyrrolidone (pvp) in chilled condition. Healthy leaves were used as the control. The homogenate was centrifuged at 5000 rpm for 10 minat 4° C (HetichZentrifugen). 100 µl of antigen was dispensed in to immunological plate (Tarson Ltd). The treatments were replicated twice and incubated for 1 h at 37°C. Later these wells were washed with phosphate buffer saline tween (PBS-T) three times at 3min interval by using ELISA plate washer (pw-40, BIORAD). Blocking was done with 100 µl of (5%)spray dried milk (SDM) for 1h at 37°C. After incubation,the plate was washed with PBS- T as done earlier. Then the plate was treated with 100 µl of polyclonal antibody(DSMZ Pvt. Ltd., Germanay) at 1: 200 dilutions in (PBS-TPO)polyvinyl pyrolidoneovalbumin. Three replications were maintained for each treatment and incubated the plate overnight under refrigerator conditions at 4°C. The plate was washed with PBS-T and treated with 100 µl of secondary antibody (DSMZ Pvt. Ltd., Germany) diluted in PBS-TPO and incubated for 2h at 37°C.The wells were washed with PBS-T with ELISA washer. Thesubstrate para nitro phenyl phosphate (pNPP) in diethanolamine buffer (1 mg per 1ml) was added to each well (100 µl per well) and incubated for 10-15 min at 37°C. Reaction was stopped by adding 50 µl of 4 per cent sodium hydroxide.

The absorbance was read at 405nm in an ELISA reader (Microplate Reader 680, BIORAD)(Appendix II).

3.2.1.2 DAS-ELISA - ***Double antibody sandwich-Enzyme linked immunosorbent assay***

Double antibody sandwich- Enzyme linked immunosorbent assay (DAS-ELISA) was done for the detection of virus causing katte disease. Firstly,*Potato virus Y* (PVY) primary antibody at 1:500 dilution was added to the wells in ELISA plate with coating buffer. The plate was incubated for 2 h at 37°C in an incubator. Later these wells were washed with phosphate buffer saline tween (PBS-T) three times at 3min interval by using ELISA plate washer (pw-40, BIORAD). One gram of the infected leaf of small cardamom was homogenized in 5 ml of coating buffer (carbonate buffer) that contains 2 per cent (w/v) polyvinyl pyrrolidone (pvp) in chilled condition.The homogenate was centrifuged at 5000 rpm for 10 min at 4° C (HetichZentrifugen). 100 µl of antigen was dispensed in to Tarson immunological plates Incubated for 2 h at 37°C in an incubator and washed with PBS-T as earlier. Secondary conjugated antibody with 1:1000 dilutions was added to the plates; incubated for 2h at 37°C wells and were washed with PBS-T with ELISA washer. The substrate para nitro phenyl phosphate (pNPP) in diethanolamine buffer (1 mg per 1ml) was added to each well (100 µl per well) and incubated for 10-15 min at 37°C. Reaction was stopped by adding 50 µl of 4 per cent sodium hydroxide.

Table 1. *Potato Virus Y*(PVY) antibody dilutions used for DAS-ELISA for the detection of katte disease of small cardamom

Sl. No.	**Polyclonal antibodies**	**Dilution**
1	Primary antibody	1:500
2	Secondary conjugated antibody	1:1000

Table 2. *Potato Virus Y* (PVY) and *Banana bract mosaic virus* (BBrMV) antibody dilutions used in DAC-ELISA for the detection of katte and chlorotic streak disease of small cardamom

Sl. No	Polyclonal antibodies	Dilution
1	PVYprimary antibody	1:200
2	PVY secondary conjugated antibody	1:10000
3	BBrMVprimary antibody	1:200
4	BBrMV secondary conjugated antibody	1:10000

3.2.2 Dot immunobinding assay (DIBA)

DIBA was carried out to detect the presence of viruses in the infected small cardamom leaves. Polyclonal antibodies of the viruses were used for the study.

One gram tissue was extracted in 1:10 antigen extracting buffer and filtered through cheese cloth. Extract of 0.8 ml was taken in an eppendorf tube to which 0.4 ml chloroform was added. The mixture was vortexed and centrifuged at 12,000 rpm for 2 min. The clarified sap was mixedwith antigen extraction buffers at 1: 4 ratio and vortexed. Nitro cellulose membrane (NCM) in squares of 1 cm X 1 cm was floated in tris buffer saline (TBS) and air dried. The sample 10 µl was spotted at the centre of each square and allowed to dry.Treated nitro cellulose membrane was immersed in blocking solution of spray dried milk powder (SDM 5%) with gentle oscillation for 1 h at room temperature.NCM was treated with TBS for 10 min and incubated overnight at 4° C in crude antiserum diluted (1; 200) in TBS-SDM.NCM was again washed in TBS for 10 min and incubated for 1 h at room temperature in secondary antibody (antirabbit IgG alkaline phosphate conjugate was dilutedat 1:10000 in TBS-SDM). After rinsing in TBS for 10 min,

NCM was incubated in a solution of nitro blue tetrazolium salt (NBT) and bromo chloro indoyl phosphate (BCIP) at room temperature in the dark for colour development. NCM was rinsed in fixing solution for 10 min after the colour development and then air dried betweenWhatman filter paper number 1 sheets and stored (Appendix III). The colour development was analysed in Gel Doc system (Gel Doc^{TM} XR+).

3.2.3Electron Microscopy

Electron microscopy studies were carried out using crude tissue extract from the severely infected leaf samples. The tissue homogenate was ground by taking 50-200 mg of leaf sample with 50-200 µl of 0.01M phosphate buffer(pH 7.0) using pestle and mortar. Fifty microlitre tissue homogenate was placed on a parafilm and a carbon coated copper grid was floated over the drop with its film side down and incubated in a humid petri dish at room temperaturefor 30 min. After incubation the grid was washed with distilled water and immediately floated on a drop of 2 per cent uranyl acetate for 30 seconds. Excess stain was drained from the grid, air dried and examined underJEOL-JEM 100 SX transmission electron microscope.

3.3 MOLECULAR CHARACTERIZATION

Molecular characterization is an important step for detection of the presence of virus which is an emerging practice for crop protection system.

Fifty milligram tissue was ground in 5 µl (denaturing solution) containing tris buffer and 0.5 per cent sodium sulphite (w/v), β mercaptoethanol (5 µl), 0.5 per cent sodium sulphite (50 µl).500 µl of 2M sodium acetate pH 4was added to the homogenised mixture and mixed well.500 µlof water saturated phenol was added and mixed well for 3-10 minutes. 100 µl of chloroform : isoamyl alcohol (24:1) was added gently and mixed by inversion.The mixture was shoke vigorously for 10 seconds, incubated on ice for 15 minand centrifuged at 12,000 rpm for 15 minutes at 4° C. Aqueous phase was collected in anew tube.DNase treatment was done by adding of chloroform : isoamyl alcohol (24:1)

and incubated for 30 min at 37° C.One volume of ice cold isopropanol was added and mixed gently by pipetting.After incubating at -80° C for 1 h, it was centrifuged at 12000 rpm for 15 min.The pellet was washed with 75 per cent ethanol and againcentrifuged at 12,000 rpm for 5 min.Pellet was air dried, dissolved in 50 µl of nuclease free water and incubated at 80°C in a water bath for 10 minfor denaturation and shifted to ice immediately for 5 min.

3.3.1Agarose gel electrophoresis

For the confirmation of the presence of RNA, gel electrophoresis was done by adding 0.6 per cent of agarose to 50 ml of 1X TAE buffer with 1.8 µl of ethidium bromide to the horizontal gel electrophoresis unit(Hoefer Power Pack, Germany). 100 bp ladder (GeNei) was used as molecular marker. Two µl of RNA was mixed with 8 µl of 6X loading dye and dispensed into the wells in the gel. The gel was run at $5Vcm^{-1}$ in TAE buffer. Gel was removed when the dye reached three fourth distanceof gel, and RNA was visualized in a UV trans illuminator system(Bio-Rad) and documented in Gel Doc system (Gel Doc ™ XR+)(Appendix V).

3.3.2Synthesis of cDNA

Total RNA was isolated from the diseased leaves and was converted to cDNA usingcDNA synthesis kit (Thermofisher). RT-PCR was done with primers specific for coat protein amplification as listed in (Table 3). After adding all the components listed in (Appendix III), the mixture was kept in thermocycler for converting it to cDNA product.

3.3.3 RT-PCR

After synthesis of cDNA, it is subjected to RT-PCR as per the protocol outlined in (Appendix IV).

The reaction mixture was run in a thermocycler (GeneAmp PCR System 9700, Applied Biosystems) under PCR conditions maintained for the amplification of coat protein of potyviruses were listed in Table 3.

Table 3. Primers specific to coat protein region of the virus used for the amplification of *Cardamom mosaic virus* (CdMV) and *Banana bract mosaic virus* (BBrMV)

Sl. No.	Primers
CdMV- F	5'CACCGCTTGCACCAATGAC 3'
CdMV- R	5'GAAAACCCACAAAAACTCCC 3'
BBrMV- F	5′ TCTGGAACG GAGTCAAC 3'
BBrMV- R	5′ GCACACATAAT TATAGGGAG 3'

(Bhat *et al.*, 2018)

Table 4. Polymerase Chain Reaction (PCR) conditions set for the amplification of coat protein of CdMV and BBrMV infecting small cardamom

Event	Temperature	Time	Cycles
cDNA	42° C	45 min	1 cycle
Initial denaturation	94° C	30 sec	35 cycles
Annealing	50° C	40 sec	
Extension	72° C	1 min	
Final Extension	72° C	10 min	1 cycle

(Bhat *et al.*, 2018)

3.3.3.1*Agarose Gel electrophoresis for PCR products*

The PCR products were checked by adding them to the gel prepared by 1.2 per cent agarose added to the 0.5X TAE buffer containing 0.5 µg/ml ethidium bromide. 2 µl of 6X loading dye was mixed with 10 µl of PCR products and were loaded on to the gel. Electrophoresis was performed at 70 V for about 1-2 h and the gel was screened under the Gel documentation system (Bio-Rad).

3.3.4. *Sequencing and BLAST analysis*

Sequence obtained after sequencing were subjected to BLASTn analysis which showed homologous series that matched with the isolated samples on National Centre for Biotechnological Information (NCBI) site.Similar sequences were aligned in MEGA 6.0 software to construct the phylogeny tree.

3.4 VIRUS ELIMINATION THROUGH MERISTEM TISSUE CULTURE

3.4.1 Source of explants

Infected samples were brought from the Idukki district. The nutrient medium developed by Murashige and Skoog (1962) was used as basal medium.

Stock solutions of macronutrients, micronutrients, vitamins and hormones were prepared as required for MS medium. Hormones like Benzyl Amino Purine (BAP), Kinetin (Kn), Indole Acetic Acid (IAA) and coconut water as a source of cytokinin were used.

3.4.2 Preparation of sterile tissue

Rhizomesinfected with both the diseases, CdMV and BBrMV were collected from field. The rhizomes were 2-3 months old and washed under running tap water and treated with copper oxy chloride @0.2% for 30 min. The rhizome was treated with 0.2 per cent Bavistin, where the outer layers were removed and washed with 2 per cent laboline soap solution. Rhizomes having 5mm diameter and 6 cm in length were selected and treated with antibacterial solution like tetracycline(K-Cycline) for about 30 min. Surface sterilization was

done with 0.1 per cent mercuric chloride for three min for three times. This was followed by soaking in 1% sodium hypochloride solution for 5min and washed three times in sterile water. The rhizomes were cut into size of 2-3 mm before placing into medium.

3.4.3Dissection and explant preparation

By using sterilized or autoclaved forceps and scalpel, the outer layers of rhizomes were taken out. The rhizomes were cut while washing with different agents. The meristem explants were of size 2 mm, entire practice was done under laminar airflow chamber.

3.4.4 Inoculation of explants

The meristem explants were transferred on to the culture bottles with medium in which $1/3^{rd}$ part of the explant was placed into the media.

3.4.5 Incubation of cultures

The explants inoculated into the culture medium were kept in a tissue culture lab where the temperature was about 24°C with a light and dark period of 12 h each.

3.4.6 Initiating media

Murashige and Skoog medium (MS medium) stock solutions were prepared as shown in (AppendixIV) and added with BAP 0.5 mgL^{-1}, Kn 0.5 mgL^{-1}, IAA2 $mgL^{-1}$and coconut water 10 per cent. Finally, agar was added to solidify the medium.

3.4.7 Multiplication media

MS medium with BAP 2 mgL^{-1}, Kn0.8mgL^{-1}, IAA 4$mgL^{-1}$and coconut water 10 per cent along with 0.5 per cent activated charcoal was used.

3.4.7 Rooting media

MS medium with IAA 1 $mgL^{-1}$and 0.5 per cent of charcoal was used. Charcoal acts as activator and removes phenol exudates.

3.4.8 Virus indexing for tissue culture plants

The absence of virus in the multiplying meristem was tested using ELISA and molecular detection was carried out in PCR.

Results

4. RESULTS

Virus infection in small cardamom has been always a threat to the cultivation of this crop in Kerala. So, the present study was undertaken to assess the occurrence of the viral diseases, characterize it and to produce disease free plants. The study was undertaken in the Department of Plant Pathology, College of Agriculture, Vellayani during the period from 2017-2019. The results of the study are detailed here.

4.1 OCCURENCE AND DISTRIBUTION OF VIRUSES IN MAJOR AREAS OF SMALL CARDAMOM CULTIVATION.

4.1.1 Survey and collection of virus infected small cardamom from Idukki

4.1.1.1. *Disease Incidence*

Survey for viral diseases in small cardamom was conducted during November 2018 to May 2019 in two major cardamom cultivated blocks in Idukki district Kattappana and Nedumkandam. Three panchayats were selected from each block. From Kattappana block Kattappana, Pampadumpara and Ambalapara panchayats were selected; and from Nedumkandam block Nedumkandam, Chembalam and Thukkupalam panchayats were selected. During the survey it was observed that Njallani variety was the most popular variety cultivated for its higher economic yield compared to other varieties.

Symptoms like discontinuous parallel dark and light green lines from mid-vein extended up to margins of leaves were observed. White and green colour mosaic patterns were also observed on pseudostem for katte disease (Plate 3) and discontinuous or continuous chlorotic streaks on leaves and spindle shaped marks on pseudostem in case of chlorotic streak disease on infected plants (Plate 4).

As a part of survey, the disease incidence was calculated from the surveyed panchayats. The incidence of katte disease was 28.40 per cent in Kattappana, 43.00 per cent in Pampadumpara, 31.25 per cent in Nedumkandam,

Plate a. Kattappana **Plate b. Pampadumpara** **Plate c. Nedumkandam**

Plate d. Ambalapara **Plate e. Thukkupalam** **Plate f. Chembalam**

Plate 3. Katte disease affected small cardamom leaf samples collected from six selected panchayats of Idukki district

Plate a. Kattappana **Plate b. Pampadumpara** **Plate c. Nedumkandam**

Plate 4. Chlorotic streak disease infected small cardamom samples (BBrMV) collected from three different panchayats of Idukki district

8.33 per cent in Thukkupalam, 3.75 per cent in Ambalapara and 5.00 per cent in Chembalam (Table 5). The disease incidence of chlorotic streak disease recorded was 41.00 per cent in Kattappana, 30 per cent in Pampadumpara and 8.33 per cent in Nedumkandam panchayats.

Highest disease incidence of katte was observed from Pampadumpara as 41.00 per cent and the lowest incidence from Ambalapara as 3.75 per cent. While incidence for chlorotic streak disease was higher in Kattappana, as high as 41.00 per cent and lower in Nedumkandam as 8.33 per cent (Figure 1).

Aphids act as vector for transmission of viruses. The leaf sheaths attached were opened to see for the presence of aphids but were not present (Plate 5).

Collateral hosts, the survival hosts of viruses were suspected as *Colocasia* spp. and *Alpinia* spp. as were present surrounding in the infected plantations. *Colocasia* spp. were found surrounding the katte infected plantations. *Alpinia* spp. were found surrounding the chlorotic streak disease infected plantations. But no symptoms of viral infection were observed in these plants (Plate 6).

4.1.2 Maintenance of virus infected cardamom from Idukki

Rhizomes of cardamom plants of Njallani variety infected with katte and chlorotic streak disease were collected from farmers field; these plants were maintained in insect proof green house at Cardamom Research Station, Pampadumpara in Idukki district (Plate 7).

4.1.3 Symptomatology

Katte infected plants expressed symptoms like light and dark green continuous lines starting from midvein region and extending up to midribs of the leaf (Plate 8 and 9). On the pseudostem, mosaic patches with white colour, extended white colour mosaic pattern and green colour mosaic patches were

Table 5. Disease incidence for katte and chlorotic streak diseases of small cardamom from thesurvey locations in Idukki district

Panchayat	Disease incidence of katte (%)	Disease incidence of chlorotic streak disease(%)
Kattapana	28.40	41.00
Pampadumpara	43.00	30.00
Ambalapara	3.75	-
Nedumkandam	31.25	8.33
Thukkupalam	8.33	-
Chembalam	5.0	-

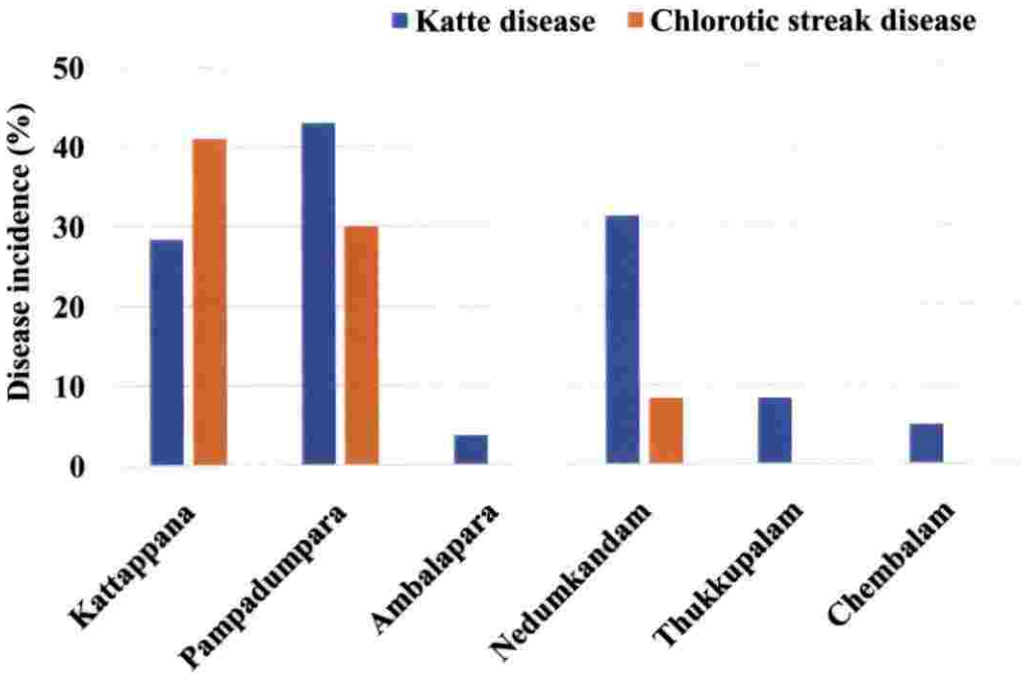


Figure 1.Disease incidence of katte and chlorotic streak diseases of small cardamom from different panchayats of Kattappana and Nedumkandam blocks of Idukki district

Plate 5. Leaf sheath of katte infected small cardamom opened to record the presence of aphids

Plate a. ***Colocasia spp.*** **Plate b.** ***Alpinia spp.***

Plate 6. Weed and other zingiberaceous plants found growing near small cardamom plantations in Kattappana and Nedumkandam blocks of Idukki district

Plate a. Cardamom variety PV 3

Plate b. Green gold

Plate 7. Small cardamom plants with katte and chlorotic streak diseases maintained in insect proof condition at Cardamom Research Station, Pampadumpara

Plate a. Alternate pale and dark green stripes along mid veins

Plate b. Chlorotic flecks developing along mid veins

Plate 8. Symptoms of kattedisease of small cardamom caused by CdMV onthe infected leaves

Plate 9. Discontinuous green stripes along veins expressed in leaves with katte disease of small cardamom caused by CdMV

observed (Plate11 and 12). In case of severe infection stunted tillers were produced by the infected plants (Plate 10).

Chlorotic streak disease infected samples expressed symptoms likethe discontinuous and continuous chlorotic flecks on the leaves and spindle shaped mosaic pattern on the stem were observed (Plate 13 and Plate 14). During the survey, it was observed that the katte infected plants could be observed year round in the plantation, while chlorotic disease was observed only during November to February month.

4.2. IMMUNO MOLECULAR CHARACTERIZATION OF THE VIRUSES

4.2.1 Enzyme linked immunosorbent assay

Detection of viruses infecting small cardamom was carried out using serological and nucleic acid based techniques. Reverse transcription polymerase chain reaction (RT-PCR) was employed for detection of the viruses at molecular level. Serological techniques like Direct antigen coating-Enzyme linked immuno sorbent assay (DAC- ELISA),Double Antibody Sandwich-Enzyme linked immuno sorbent assay (DAS- ELISA) and Dot immunobinding assay (DIBA) was used to detect the *Cardamom mosaic virus* (CdMV) that causes katte disease by using polyclonal antibody of *Potato virus Y* (PVY) virus since CdMV belongs to PVY group (Plate 15). All the reactions gave positive result. The primary antibody at 1:500 and the secondary conjugated antibody at 1:1000dilutionswere used.

The mean value of absorbance (OD value) recordedwas0.655 for katte disease infected plants compared to the mean OD value of 0.226 recorded in the healthy samples (i.e. 2.9 times higher)which were collected from Kattappana panchayat (Table 6). From Pampadumpara an OD value of 1.388 was recorded in the infected plants against the OD value of 0.336 in the healthy plants, which was 6.1fold more (Table 7). The infected samples from Nedumkandam recorded an OD value of 0.990 against 0.337 (in the healthy plants) and there was 2.6fold increase in the mean absorbance value (Table 8).

Plate 10. Severe stunting of tillers in katte disease infected small cardamom plants

Plate a. Alternate light and dark green bands

Plate b. Chlorotic flecks along the midrib to margin

Plate c. Mosaic symptom on young leaf

Plate 11. Symptoms of katte disease infection in small cardamom plants

Plate a. Pseudostem with white colour mosaic patterns

Plate b. Pseudostem with enlarged mosaic pattern

Plate c. Pseudostem with green colour mosaic pattern

Plate 12. Katte disease infected pseudostem of small cardamom with varying mosaic pattern

Plate a. Chlorotic streaks on the leaves

Plate b. Spindle shaped mark on the pseudostem

Plate 13. Symptoms on small cardamom leaves and pseudostem infected with chlorotic streak disease caused by BBrMV

Plate a.
Kattappana
isolate

Plate b.
Pampadumpara
isolate

Plate c.
Nedumkandam
isolate

Plate a.
Kattapana
isolate

Plate b.
Pampadumpara
isolate

Plate c.
Nedumkandam
isolate

Plate 14. Different symptoms of chlorotic streak disease caused by BBrMV in small cardamom from the surveyed locations of Idukki distict

Table 6. Reaction of CdMV to polyclonal antibody PVY in DAC-ELISA for samples collected from Kattappana panchayat

Samples	Absorbance value at 405 nm (*)	Fold increase of OD value	Reaction
Healthy	0.226	-	-
Kattappana 1	0.486	2.1	+ ve
Kattappana 2	0.572	2.6	+ ve
Kattappana 3	0.655	2.9	+ ve
Kattappana 4	0.456	2.0	+ ve
Kattappana 5	0.448	2.0	+ ve
Kattappana 6	0.486	2.1	+ ve
Kattappana 7	0.515	1.8	+ve
Kattappana 8	0.550	1.9	+ ve
Kattappana 9	0.498	2.2	+ ve
Kattappana 10	0.468	2.0	+ ve

+ = Positive reaction

* OD value calculated by taking mean of 3 replications

Table 7. Reaction of katte diseasesamples infected by CdMV to polyclonal antibody of PVY using DAC-ELISA collected from Pampadumpara panchayat, Idukki

Samples	Absorbance value at 405 nm (*)	Fold increase of OD value	Reaction
Healthy	0.226	-	-
Pampadumpara 1	1.388	6.1	+ve
Pampadumpara 2	1.181	5.2	+ve
Pampadumpara 3	1.134	5.0	+ve
Pampadumpara 4	1.178	5.2	+ve
Pampadumpara 5	1.218	5.3	+ve
Pampadumpara 6	0.860	3.8	+ve
Pampadumpara 7	1.309	5.7	+ve
Pampadumpara 8	1.300	5.7	+ve
Pampadumpara 9	1.166	5.1	+ve
Pampadumpara 10	1.178	5.2	+ve

+ = Positive reaction

* OD value calculated by taking mean of 3 replications

Table 8. Reaction of katte disease samples infected by CdMV to polyclonal antibody PVY using DAC-ELISA collected from Nedumkandam panchayat, Idukki

Samples	Absorbance value at 405 nm (*)	Increase of OD value	Reaction
Healthy	0.377	2.2	+ ve
Nedumkandam 1	0.845	2.2	+ ve
Nedumkandam 2	0.855	2.2	+ ve
Nedumkandam 3	0.806	2.1	+ ve
Nedumkandam 4	0.919	2.4	+ ve
Nedumkandam 5	0.778	2.0	+ ve
Nedumkandam 6	0.796	2.1	+ ve
Nedumkandam 7	0.802	2.1	+ ve
Nedumkandam 8	0.990	2.6	+ ve
Nedumkandam 9	0.821	2.1	+ ve
Nedumkandam 10	0.801	2.1	+ ve

+ = Positive reaction

* OD value calculated by taking mean of 3 replications

Plate 15. ELISA plate showing reaction of CdMV to the polyclonal antibody PVY in DAS-ELISA

H- Healthy sample of small cardamom

D- Katte diseased affected sample from leaf

B- Katte diseased affected sample from bract

The highest OD value observed from Ambalapara panchayat was 0.654for katte infected plants compared to 0.179 for the healthy samples i.e. 3.6 fold increase of OD value in the infected samples (Table 9). The infected samples from Thukkupalam panchayat recorded 0.908 against 0.336 in healthy which showed 2.7fold increase in the OD value (Table 10) and from Chembalam panchayat the OD value of the infected sample recorded was 1.388 against 0.531 for the healthy samples which was 2.6 fold more (Table 11).

Direct antigen coating (DAC-ELISA) with polyclonal antibody of *Banana bract mosaic virus* (BBrMV) was undertaken for the detection of chlorotic streak disease. The primary antibody dilution was 1:200 and secondary conjugated antibody dilution used was 1:10000 (Plate 16). All the reactions were positive from all the survey regions. The highest mean value of absorbance was 1.079 in the infected sample and 0.392 for the healthy sample which was 2.6 fold more from the Kattappana samples (Table 12), from Pampadumpara 0.910 against the healthy 0.408 which was 2.2 fold more (Table 13), and from Nedumkandam the value of 0.902 against the healthy 0.408was recorded, which was 2.2 fold more (Table 14).

4.2.2 Dot immunobinding assay

DIBA is one of the best and simple methods for the detection which can be done at the field level. DIBA was done to detect the presence of katte and chlorotic streak disease in the collected samples by using two polyclonal antibodies, PVY and BBrMV respectively. PVY antiserum was used at the dilution of 1: 200 for primary antibody and 1:1000 for secondary antibody; and BBrMV primary antibody was used at 1:500 whereas 1:1000 for secondary conjugated antibody. Dark purple colour development in the infected sample indicating the presence of virus in the sample and was subjected to gel documentation (BIORAD Molecular Imager GEL DOC^{TM} XR+)(Plate 17).The results revealed that the infected sample had high mean value of intensity compared to healthy sample(Table15 &16).

Table 9. Reaction of katte disease samples infected by CdMV to polyclonal antibody of PVY using DAC-ELISA collected from Ambalapara panchayat

Samples	Absorbance value at 405 nm (*)	Increase of OD value	Reaction
Healthy	0.179	-	-
Ambalapara 1	0.386	2.1	+ve
Ambalapara 2	0.513	2.8	+ve
Ambalapara 3	0.410	2.2	+ve
Ambalapara 4	0.461	2.5	+ve
Ambalapara 5	0.445	2.4	+ve
Ambalapara 6	0.383	2.1	+ve
Ambalapara 7	0.654	3.6	+ve
Ambalapara 8	0.482	2.6	+ve
Ambalapara 9	0.506	2.8	+ve
Ambalapara 10	0.516	2.8	+ve

+ = Positive reaction

* : OD value calculated by taking mean of 3 replications

Table 10. Reaction of katte disease samples infected by CdMV to polyclonal antibody of PVY using DAC-ELISA collected from Thukkupalam panchayat, Idukki

Samples	Absorbance value at 405 nm (*)	Fold increase of OD value	Reaction
Healthy	0.336	-	-
Thukkupalam 1	0.882	2.6	+ve
Thukkupalam 2	0.672	2.0	+ve
Thukkupalam 3	0.880	2.6	+ve
Thukkupalam 4	0.735	2.2	+ve
Thukkupalam 5	0.777	2.3	+ve
Thukkupalam 6	0.778	2.3	+ve
Thukkupalam 7	0.908	2.7	+ve
Thukkupalam 8	0.841	2.5	+ve
Thukkupalam 9	0.792	2.3	+ve
Thukkupalam 10	0.850	2.5	+ve

+ = Positive reaction

* OD value calculated by taking mean of 3 replications

Table 11. Reaction of katte disease samples infected by CdMV to polyclonal antibody of PVY using DAC-ELISA from Chembalam panchayat Idukki

Samples	Absorbance value at 405 nm (*)	Fold increase of OD value	Reaction
Healthy	0.531	-	-
Chembalam 1	1.388	2.6	+ve
Chembalam 2	1.178	2.2	+ve
Chembalam 3	1.181	2.2	+ve
Chembalam 4	1.134	2.1	+ve
Chembalam 5	1.178	2.2	+ve
Chembalam 6	1.218	2.2	+ve
Chembalam 7	1.128	2.1	+ve
Chembalam 8	1.156	2.1	+ve
Chembalam 9	1.145	2.1	+ve
Chembalam 10	1.167	2.1	+ve

+ = Positive reaction

* OD value calculated by taking mean of 3 replications

Table 12. Reaction of chlorotic streak disease samples infected by BBrMV to polyclonal antibody of BBrMV in DAC-ELISA collected from Kattappana panchayat, Idukki

Samples	Absorbance value at 405 nm (*)	Fold increase of OD value	Reaction
Healthy	0.392	-	-
Kattappana 1	1.094	2.7	+ ve
Kattappana 2	1.039	2.6	+ ve
Kattappana 3	0.841	2.1	+ ve
Kattappana 4	0.819	2.0	+ ve
Kattappana 5	0.789	2.0	+ ve
Kattappana 6	1.010	2.5	+ ve
Kattappana 7	0.825	2.1	+ve
Kattappana 8	0.879	2.2	+ve
Kattappana 9	0.858	2.1	+ve
Kattappana 10	0.862	2.1	+ve

+ = Positive reaction

* : OD value calculated by taking mean of 3 replications

Table 13. Reaction of chlorotic streak disease samples infected by BBrMV to polyclonal antibody of BBrMV in DAC-ELISA collected from Pampadumpara panchayat, Idukki

Samples	Absorbance value at 405 nm (*)	Fold increase of OD value	Reaction
Healthy	0.408	-	
Pampadumpara 1	0.822	2.0	+ve
Pampadumpara 2	0.817	2.0	+ve
Pampadumpara 3	0.910	2.2	+ve
Pampadumpara 4	0.887	2.1	+ve
Pampadumpara 5	0.863	2.1	+ve
Pampadumpara 6	0.862	2.1	+ve
Pampadumpara 7	0.854	2.0	+ve
Pampadumpara 8	0.832	2.0	+ve
Pampadumpara 9	0.842	2.0	+ve
Pampadumpara 10	0.870	2.1	+ve

+ = Positive reaction

* OD value calculated by taking mean of 3 replications

Table 14. Reaction of chlorotic streak disease samples infected by BBrMV to polyclonal antibody of BBrMV in DAC-ELISA collected from Nedumkandam panchayat, Idukki

Samples	Absorbance value at 405 nm (*)	Fold increase of OD value	Reaction
Healthy	0.408	-	-
Nedumkandam 1	0.826	2.0	+ve
Nedumkandam 2	0.862	2.1	+ve
Nedumkandam 3	0.897	2.1	+ve
Nedumkandam 4	0.877	2.1	+ve
Nedumkandam 5	0.902	2.2	+ve
Nedumkandam 6	0.842	2.0	+ve
Nedumkandam 7	0.862	2.1	+ve
Nedumkandam 8	0.864	2.1	+ve
Nedumkandam 9	0.870	2.1	+ve
Nedumkandam 10	0.820	2.0	+ve

+ = Positive reaction

* OD value calculated by taking mean of 3 replications

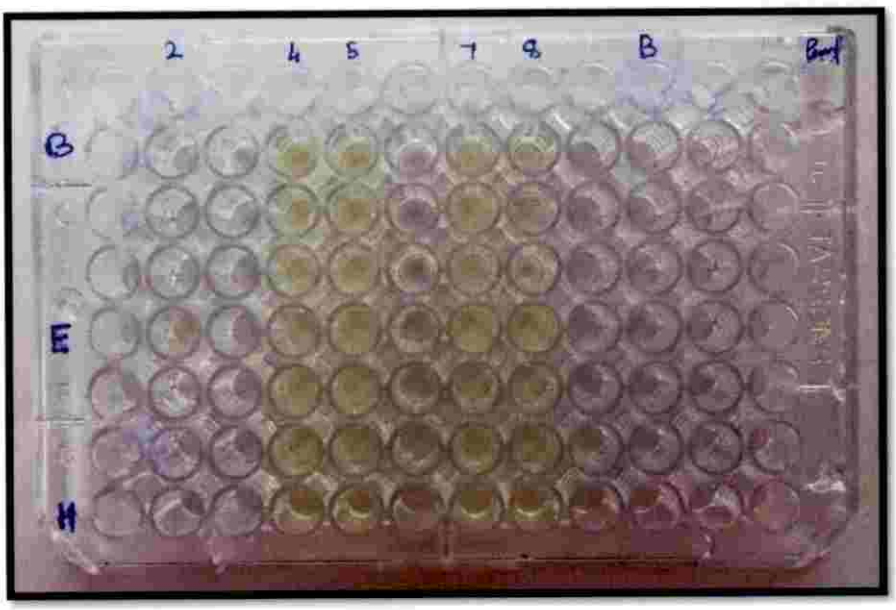


Plate 16. ELISA plate showing reaction of BBrMV infecting small cardamom (chlorotic streak disease) against the respective polyclonal antibody in DAC-ELISA

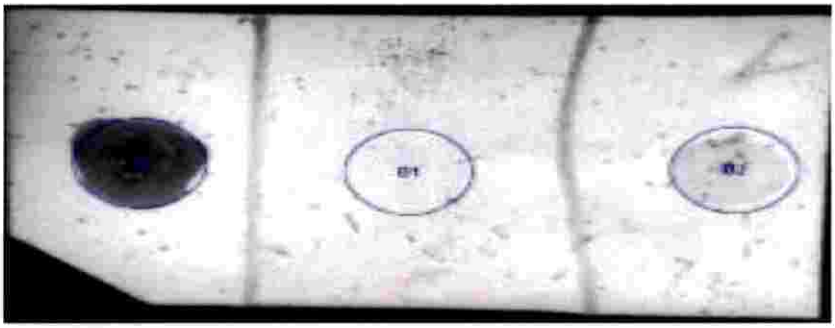
B2- Healthy leaf sample of small cardamom
E2- Healthy bract sample of small cardamom
4&5 –Chlorotic streak disease affected leaf samples
7&8- Chlorotic streak disease affected bract samples

Table 15. Reaction of katte disease infected small cardamom plants by CdMV to polyclonal antibody PVY

Sl. No.	Label	Type	Volume (Int)	Adj. Vol. (Int)	Mean background (Int)	Min value (Int)	Max Value (Int)	Mean value (Int)	Area (mm2)
1.	U1	Diseased	13,013,332	3,342,692	1,324.6	1,104	2,549	1,782.4	34.0
2.	U2	Healthy	10,330,506	787,687	1,419.9	1,084	2,013	1,537.0	31.3
3.	U3	Background	9,081,237	140,336	1,263.1	1,004	2,489	1,243.8	34.0

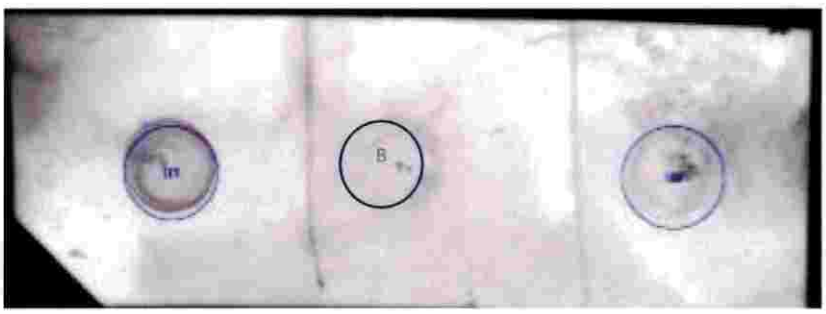
Table 16. Reaction of chlorotic mottle disease infected small cardamom plants by BBrMV to polyclonal antibody of BBrMV

Sl. No.	Label	Type	Volume (Int)	Adj. Vol. (Int)	Mean back-ground (Int)	Min value (Int)	Max Value (Int)	Mean value (Int)	Area (mm2)
1.	U1	Diseased	19,952,473	6,641,211	1,980.5	1,120	3,707	2,068.7	7 31.3
2.	U2	Healthy	8,930,304	1,098,521	1,276.	1,018	3,070	1,455.2	28.6
3.	U3	Background	7,938,770	11,846	1,291.7	1,085	2,857	1,293.6	28.6



Positive reaction for katte infected sample in DIBA

U1= Diseased; U2= Healthy; B = Background



Positive reaction for chlorotic streak infected sample in DIBA

U1= Diseased; U2= Healthy; B = Background

Plate 17. Positive reaction of CdMV and BBrMV infected samples of small cardamom to the polyclonal antibodies of PVY and BBrMV respectively in DIBA

4.2.3 Electron microscopy

Electron micrograph revealed the presence of Potyvirus as long flexuous rod with a length of 750 nm (Plate 18).

4.3 MOLECULAR DIAGNOSIS

Molecular diagnosis and detection of the viruses were done based on PCR amplification of nucleic acid of coat protein of the respective viruses. The amplification of coat protein of the viruses was done using Reverse transcription-Polymerase chain reaction (RT-PCR) using two different primers.

4.3.1 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The virus infected samples collected from different panchayats were subjected to RT– PCR. After isolating RNA and reverse transcribed to cDNA, the coat protein had been amplified using specific primers separately for two different viruses CdMV and BBrMV listed in (Table 4). Amplicon of size879 bp was obtained for katte infected samples from Kattappana region, 889 bp was obtained for katte infected samples from Pampadumpara region and 905 bp was obtained for katte infected samples from Nedumkandam region. In the case of chlorotic streak disease sample, the amplicon of 625 bp was obtained for Kattappana region sample, 626 bp for Pampadumpara region and 636 bp for Nedumkandam region samples(Plate 19).

4.3.2 BLAST analysis

On BLAST analysis, the CdMV isolate from Kattappana had shown 96.04 per cent identity with *Indian cardamom mosaic virus* isolate from Thalathamane. The CdMV isolate from Pampadumpara had shown 97.33% identity with *Indian Cardamom mosaic virus* from Thalathamane (Karnataka) (Table17) and *Cardamom mosaic virus* isolate from Appangala (Table 18). CdMV isolate from Nedumkandam had shown 96.90 per cent identity with *Cardamom mosaic virus* isolate from Appangala (Table 19).

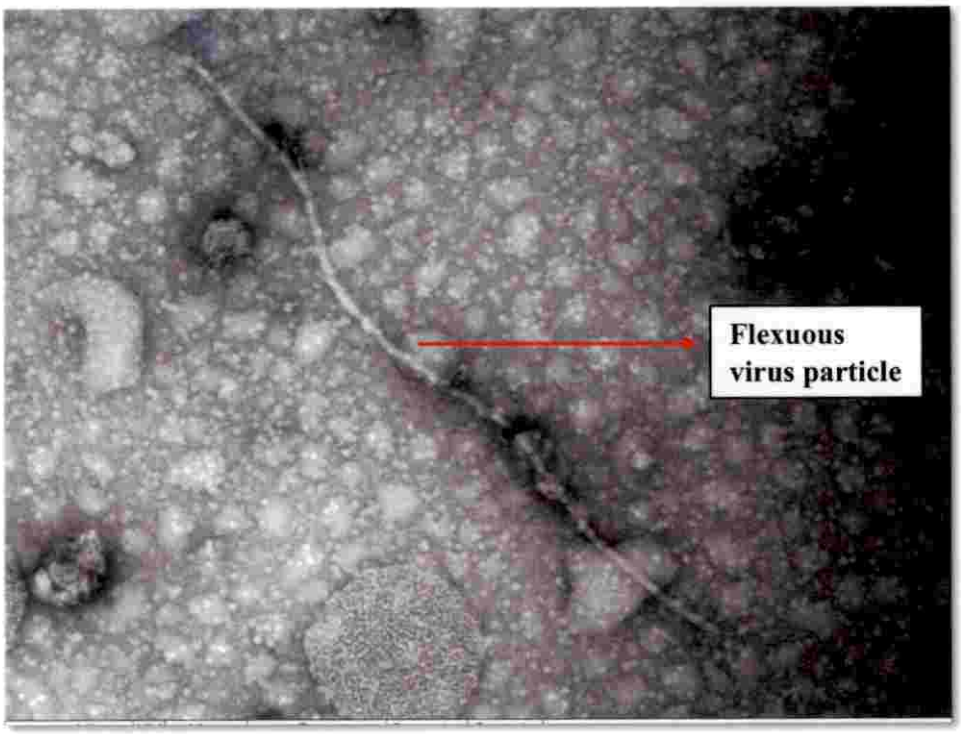


Plate 18. Electron micrograph of CdMV, causing katte disease of small cardamom, showing flexuous shaped virus particle

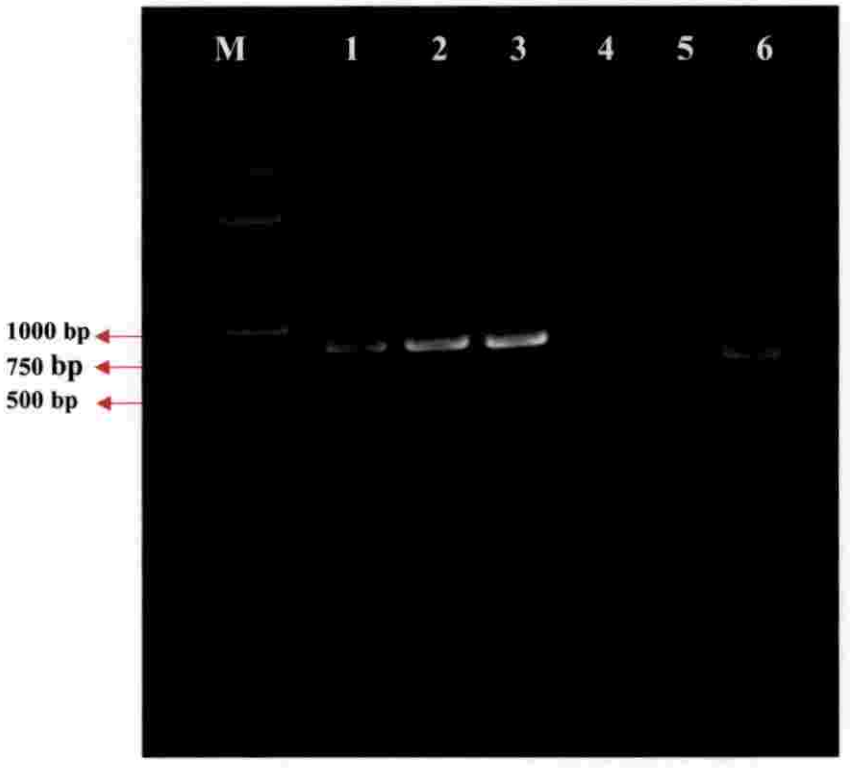


Plate 19. Electrophoresis gel image of PCR product of amplified RNA of CdMV and BBrMV infected samples collected from Idukki; lane M- 100 bp DNA ladder; 1, 2, 3 – CdMV isolates from Kattappana, Pampadumpara and Nedumkandam panchayats; lane 4, 5, 6 – BBrMV isolates from Kattappana, Pampadumpara and Nedumkandam panchayats

Table 17. NCBI BLAST analysis for sequences similarity of CdMV isolate from Kattappana Idukki with other CdMV isolates reported in cardamom

Accession number	Description	Nucleotide sequence similarity
AJ308475.1	Indian cardamom mosaic virus partial cp gene for coat protein, genomic RNA(India: Thalathamane)	96.04%
AJ308472.1	Indian cardamom mosaic virus partial cp gene for coat protein, genomic RNA, isolate from Appangala	95.92%
NC039088.1	Cardamom mosaic virus isolate KS, complete genome	95.47%
AY833735.1	Anemahal Polyprotein gene, partial cds	95.47%
AJ308474.1	Indian cardamom mosaic virus partial cp gene for coat protein, genomic RNA(India: Madikeri)	95.47%
AY823986.1	Indian cardamom mosaic virus isolate SKP-3 polyprotein gene, partial cds	95.24%
AY609386.1	Indian cardamom mosaic virus isolate Somwarpet polyprotein gene, partial cds	95.13%
AY823985.1	Indian cardamom mosaic virus isolate SKP1 polyprotein gene, partial cds	94.26%
JN544082.1	Cardamom mosaic virus isolate Sakleshpur capsid protein gene, partial cds	96.00%
AY609385.1	Indian cardamom mosaic virus isolate Margodu polyprotein gene, partial cds	85.92%

Table 18. NCBI BLAST analysis for sequence similarity of CdMV isolate from Pampadumpara, Idukki with other CdMV isolates reported in cardamom

Accession number	Description	Nucleotide sequence similarity
AJ308475.1	Indian cardamom mosaic virus partial cp gene for coat protein, genomic RNA(India: Thalathamane)	96.04%
AJ308472.1	Indian cardamom mosaic virus partial cp gene for coat protein, genomic RNA, isolate from Appangala	95.92%
NC039088.1	Cardamom mosaic virus isolate KS, complete genome	95.47%
AY833735.1	Anemahal Polyprotein gene, partial cds	95.47%
AJ308474.1	Indian cardamom mosaic virus partial cp gene for coat protein, genomic RNA (India: Madikeri)	95.47%
AY823986.1	Indian cardamom mosaic virus isolate SKP-3 polyprotein gene, partial cds	95.24%
AY609386.1	Indian cardamom mosaic virus isolate Somwarpet polyprotein gene, partial cds	95.13%
AY823985.1	Indian cardamom mosaic virus isolate SKP1 polyprotein gene, partial cds	94.26%

Table 19. NCBI BLAST analysis for sequences similarity of CdMV isolate from Nedumkandam Idukki with other CdMV isolates reported in cardamom

Accession number	Description	Nucleotide sequence similarity
AF189125.1	Cardamom mosaic virus polyprotein gene, partial cds	96.37%
AY609385.1	Indian cardamom mosaic virus isolate Margodupolyprotein gene, partial cds	93.33%
JN544081.1	Cardamom mosaic virus isolate Appangala capsid protein gene, partial cds	96.90%
AJ312774.1	Indian cardamom mosaic virus partial cp gene for coat protein, genomic RNA	81.33%
JN544078.1	Cardamom mosaic virus isolate Thadiyankudisai capsid protein gene, partial cds	79.60%

BBrMV isolate from Kattappana had shown 91.01 per cent identity with *Banana bract mosaic virus* CdM (Karnataka) (Table 20), BBrMV isolate from Pampadumpara had shown 90.29 per cent identity with *Banana bract mosaic virus* isolate I1 polyprotein (Coimbatore) (Table 21), BBrMV from Nedumkandam had shown 95.76 per cent identity with *Banana bract mosaic virus* isolate KAPP2 (Thrissur), *Banana bract mosaic virus* isolate Coimbatore and *Banana bract mosaic virus* isolate I1(Table 22).

4.3.3 Phylogeny tree

Phylogeny tree was constructed using MEGA 6.0 software (Figure 2). CdMV isolates from Kattappana and Nedumkandam clustered together while CdMV Pampadumpara was in a separate clade. BBrMV isolates from Pampadumpara and Nedumkandam were clustered together while BBrMV Kattappana was in a separate clade.

Phylogeny tree was also constructed for CdMV from Kattappana, Pampadumpara and Nedumkandam with other CdMV isolates that were collected from small cardamom grown in different parts of India (Figure 3). CdMV Kattappana and Nedumkandam clustered together while CdMV Pampadumpara was related to CdMV Thadiyankudisai, ICdMV Kattappana, CdMV Vandiperiyar.

A phylogeny tree was constructed for BBrMV isolates from Kattappana, Pampadumpara and Nedumkandam. BBrMV Kattappana was clustered withBBrMV Karnataka, BBrMV Pampadumpara and Nedumkandam were grouped together in another clade (Fig. 4).

4.4 ELIMINATION OF VIRUS THROUGH MERISTEM TISSUE CULTURE

Rhizomes were collected from the infected plants. An explant of size 2 mm (Plate 20) was inoculated on MS medium with different levels of auxin ad cytokinin. The MS media supplemented with coconut water, 2mg/ml BAP and 1 mg/ml IAA recorded better shoot initiation.

Table 20. NCBI BLAST analysis for sequences similarity of BBrMV isolate from Kattappana panchayat, Idukki with other BBrMV isolates reported in banana

Accession number	Description	Maximum Nucleotide Identity (%)
AY776327.1	Banana bract mosaic virus CdM from India polyprotein gene, partial cds	91.01%
MG758140.1	Banana bract mosaic virus isolate Wayanad, complete genome	90.84%
DQ851496.1	Banana bract mosaic virus isolate complete genome	89.28%
KT456531.1	Banana bract mosaic virus isolate BBrMV Ginger, complete genome	89.09%
EU414267.1	Banana bract mosaic virus from Philippines polyprotein gene, partial cds	88.56%
HM131454.1	Banana bract mosaic virus isolate TRY, complete genome	88.17%
AY529121.1	Banana bract mosaic virus clone bbmvcp-604 coat protein gene, partial cds; and 3' UTR	89.44%
MH703900.1	Banana bract mosaic virus isolate KAPP2 polyprotein mRNA, partial cds	89.94%
AY494979.1	Banana bract mosaic virus isolate Coimbatore polyprotein mRNA, partial cds	89.94%
AF071582.1	Banana bract mosaic virus isolate 11polyprotein mRNA, partial cds complete genome	89.94%

Table 21. NCBI BLAST analysis for sequences similarity of BBrMV isolate from Pampadumpara Idukki with other BBrMV isolates reported in banana

Accession number	Description	Nucleotide sequence similarity
AY776327.1	Banana bract mosaic virus CdM from India polyprotein gene, partial cds	88.42%
MG758140.0	Banana bract mosaic virus isolate Wayanad, complete genome	88.53%
DQ851496.1	Banana bract mosaic virus, complete genome	88.18%
AF071582.1	Banana bract mosaic virus isolate I1 polyprotein mRNA, partial cds	90.29%
MH703900.1	Banana bract mosaic virus isolate KAPP2 polyprotein mRNA, partial cds	90.08%
AY494979.1	Banana bract mosaic virus isolate Coimbatore polyprotein mRNA, partial cds	90.08%
EU531470.1	Banana bract mosaic virus isolate Coimbatore coat protein gene, partial cds	89.36%
AY529121.1	Banana bract mosaic virus clone bbmvcp-604 coat protein gene, partial cds; and 3' UTR	89.36%
KT456531.1	Banana bract mosaic virus isolate BBrMV Ginger complete genome	87.62%
EU414267.1	Banana bract mosaic virus from Philippines polyprotein gene, partial cds	87.64%

Table 22. NCBI BLAST analysis for sequences similarity of BBrMV isolate from Nedumkandam panchayat, Idukki with other BBrMV isolates reported in banana

Accession number	Description	Nucleotide sequence similarity
AY776327.1	Banana bract mosaic virus CdM from India polyprotein gene, partial cds	95.28%
MG758140.0	Banana bract mosaic virus isolate Wayanad, complete genome	94.95%
HM131454.1	Banana bract mosaic virus isolate TRY, complete genome	93.97%
DQ851496.1	Banana bract mosaic virus, complete genome	93.97%
KT456531.1	Banana bract mosaic virus isolate BBrMVGinger, complete genome	93.65%
EU414267.1	Banana bract mosaic virus from Philippines polyprotein gene, partial cds	93.66%
AY529121.1	Banana bract mosaic virus clone bbmvcp-604 coat protein gene, partial cds; and 3' UTR	95.17%
MH703900.1	Banana bract mosaic virus isolate KAPP2 polyprotein mRNA, partial cds	95.76%
AY494979.1	Banana bract mosaic virus isolate Coimbatore polyprotein mRNA, partial cds	95.76%
AF071582.1	Banana bract mosaic virus isolate I1 polyprotein mRNA, partial cds	95.76%

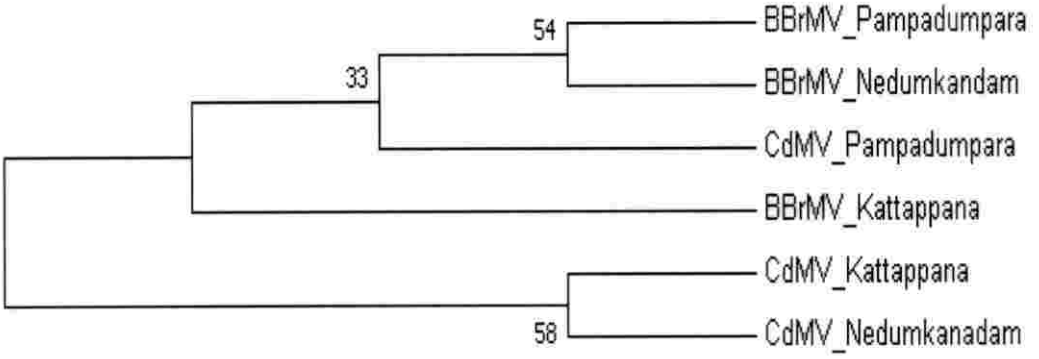


Figure 2. Phylogeny tree constructed in MEGA 6.0 by neighbour joining method showing the relationship between the different isolates of CdMV and BBrMV causing katte and chlorotic streak diseases respectively in small cardamom from Kattappana, Pampadumpara and Nedumkandam panchayats of Idukki district

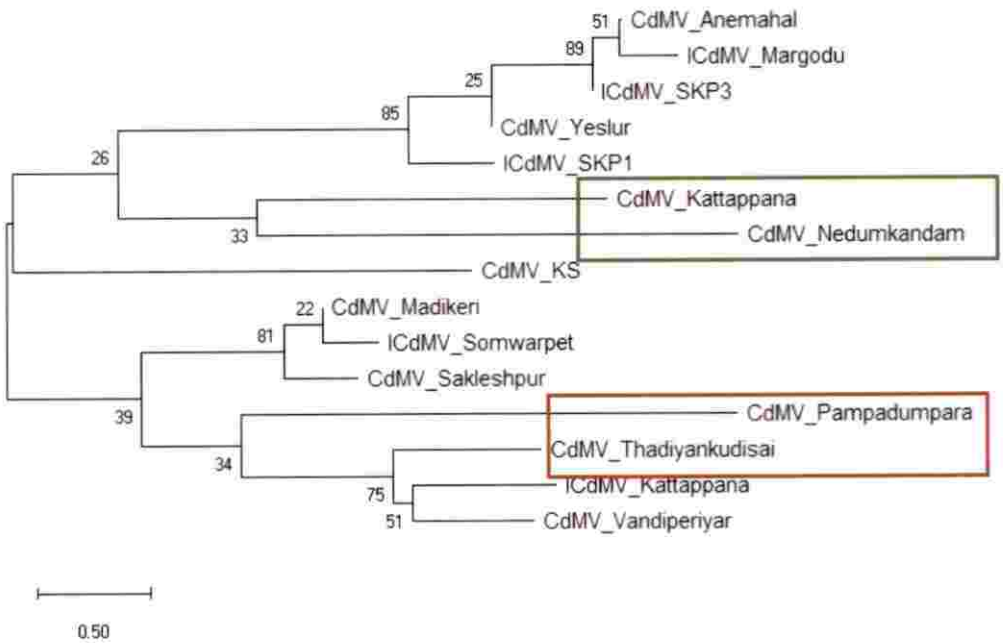


Figure 3. Phylogeny tree constructed in MEGA 6.0 by neighbour joining method showing the relationship between the different isolates of CdMV causing katte diseases in small cardamom from Kattappana, Pampadumpara and Nedumkandam panchayats of Idukki district with the sequences of CdMV reported from different small cardamom cultivated areas in India.

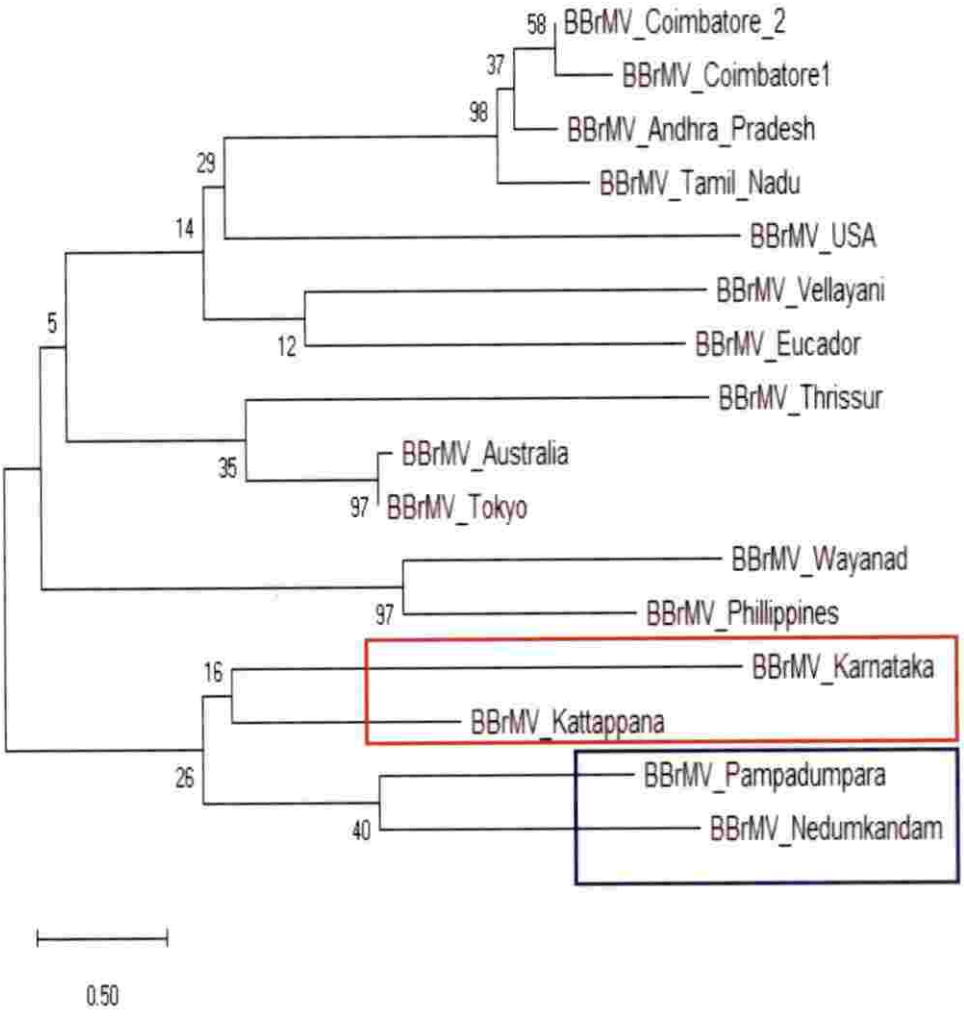


Figure 4. Phylogeny tree constructed in MEGA 6.0 by neighbour joining method showing the relationship between the different isolates of BBrMV causing chlorotic mottle disease in small cardamom from Kattappana, Pampadumpara and Nedumkandam panchayats of Idukki district with the sequences of BBrMV reported from different banana cultivated areas in India.

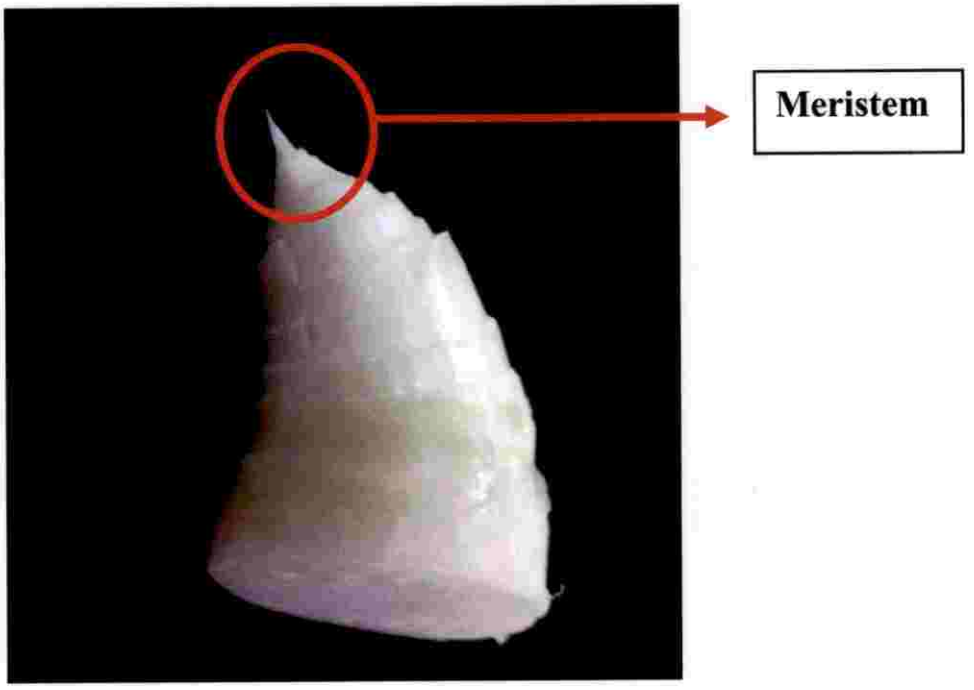


Plate 20. Meristem isolated from the virus infected small cardamom plants

The proliferation of meristems started at 30 days after inoculation (Plate 21). The buds proliferated into shoots. The shoots were changed to the rooting media containing MS media supplemented with coconut water, 3.5 mg/ml IAA and 1.5 mg/ml of BAP. The plantlets that developed roots were tested for viral diseases by ELISA test by using the polyclonal antiserum for detection of CdMV and BBrMV.

Only direct organogenesis was observed without callus phase. As there were two types of growth development stages direct method and indirect method. Direct method of growth has no callus stage, whereas indirect method has callus stage from which the organs develop. The difference between direct and indirect organogenesis was due to hormonal variations. as there is hormonal variation only direct organogenesis was observed.

DAS ELISA was done to test the meristems against the presence of the CdMV. The primary antibody dilution was 1:500 and secondary antibody dilution was 1:1000. All the reactions gave OD values on par with healthy titre, indicating that the meristems were virus free (Table 23). DAC ELISA was performed to test the meristem for the presence of BBrMV by using polyclonal antiserum with primary antibody dilution of 1:200 and secondary conjugated antibody at a dilution of about 1:10000. All the samples OD values were on par with healthy value (Table 24).

RT-PCR was performed by using specific coat protein primers. No amplicons were observed in the gel indicating the absence of viruses in the multiplying shoots (Plate 22).

Table 23. Reaction of multiplying shoots raised from the meristem tip of katte disease samples infected by CdMV using specific PVY polyclonal antibody in DAS ELISA

Samples	Absorbance value at 405 nm (*)	Fold increase of OD value	Result
Healthy	0.064	-	-
Plantlet 1	0.063	0.9	- ve
Plantlet 2	0.049	0.7	- ve
Plantlet 3	0.041	0.6	- ve
Plantlet 4	0.052	0.8	- ve

- = Negative reaction

*:OD value calculated by taking mean of 3 replications

Table 24. Reaction of multiplying shoots raised from the meristem tip of chlorotic mottle disease samples infected by BBrMV using specific BBrMV polyclonal antibody in DAC ELISA

Samples	Absorbance value at 405 nm (*)	Fold increase of OD value	Result
Healthy	0.065	-	-
Plantlet 1	0.034	0.5	- ve
Plantlet 2	0.050	0.7	- ve
Plantlet 3	0.044	0.6	- ve
Plantlet 4	0.057	0.8	- ve

- = Negative reaction

*:OD value calculated by taking mean of 3 replications

Plate a. Meristem from the virus infected sample

Plate b. Meristem transforming to shoot after subculture

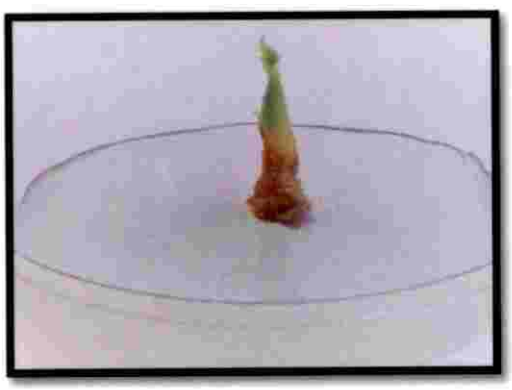


Plate c. Shoot initiation

Plate d. Root and shoot formation

Plate 21.Stages of development in meristem culture

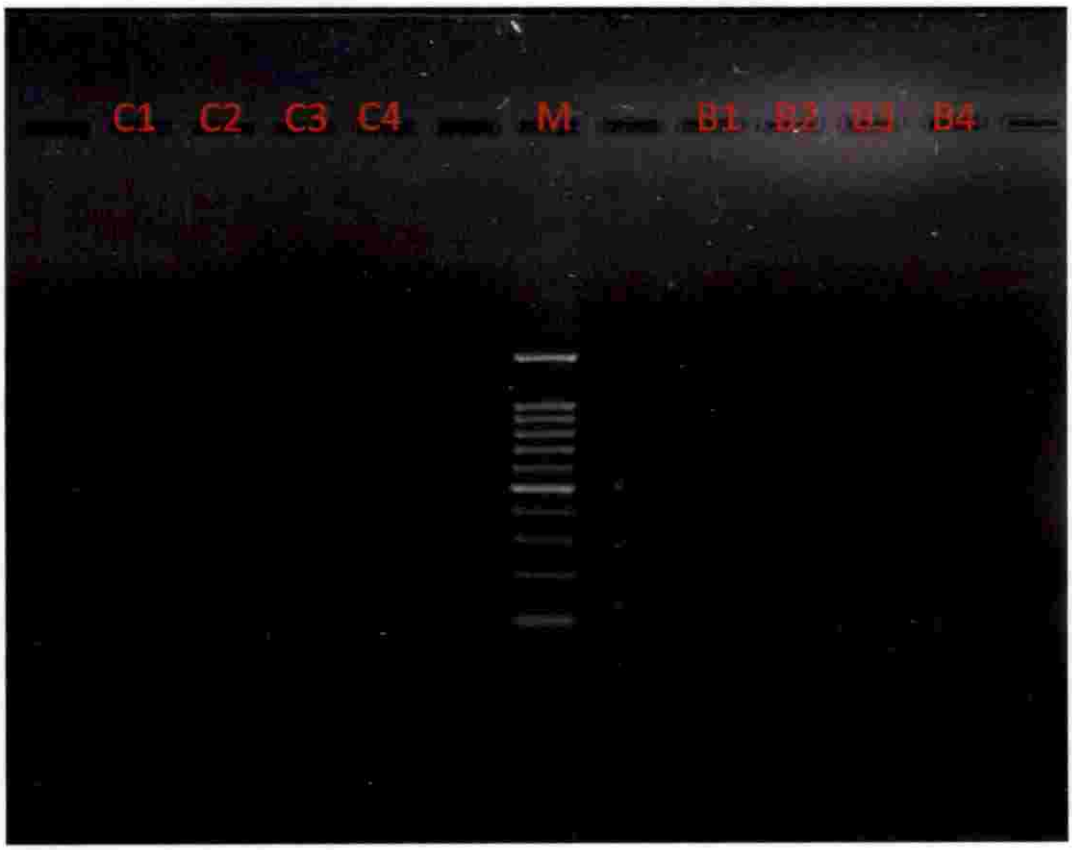


Plate 22.Electrophoresis gel image of PCR product of amplified RNAof shoots obtained from meristem culture; lane M- 100 bp DNA ladder, lane 2, 3, 4, 5 – for CdMV detection andlane 9, 10, 11, 12 –for BBrMV detection

Discussion

5. DISCUSSION

Small cardamom *Elettaria cardamomum Maton* considered as queen of spices is cultivated in the Western Ghats of India particularly in hilly areas of Tamil Nadu, Karnataka and Kerala. Viral diseases have emerged as a serious threat to the production of small cardamom. Being a high value crop, it is essential to characterise the viruses and develop an eco-friendly strategy to manage the diseases. Hence, this study was undertaken with the objectives to study the occurrence and distribution of viruses infecting small cardamom, molecular characterization of the viruses and elimination of viruses through meristem culture for the production of disease free planting material.

5.1 SURVEY

Survey was conducted during November 2018- May 2019intwo blocks of Idukki district, namely Kattappana and Nedumkandam and three panchayats from each block were selected. A total of 60plantations from Kattappana, Pampadumpara, Ambalapara, Nedumkandam, Thukkupalam and Chembalam were surveyed during November 2017-May 2019 and observed for the incidence of katte and chlorotic streak diseases. It was found that highest incidence for katte disease was recorded from Pampadumpara with 43.00 percent, while Ambalapara panchayat recorded low incidence of the disease with 3.75percent.

Surveys were conducted in order to study the distribution, incidence and economical loss due to a pest or disease in that particular area. Mayne (1951) surveyed South India to study the incidence of katte disease in small cardamom and reported that the disease incidence varied from0.01-99.00 percent.

Crop losses of 38per cent, 62per cent and 68.7per cent for the first, second and third year of infection respectively were recorded in katte disease infected small cardamom plants (Varma 1962; Venugopal and Naidu 1987; Venugopal 1995). Venugopal (1995) reported that the disease incidence ranged from 10.4 - 42.8 per cent in different small cardamom plantations infected with katte disease. In Karnataka the disease incidence of katte was reported to be 85per cent, whereas 12per cent incidence had been recorded from Devikulam taluk of Idukki district by Biju *et al.* (2010).

The incidence of chlorotic streak disease was found only in three of the six surveyed panchayats *viz*., Kattappana, Pampadumpara and Nedumkandam. The highest disease incidence was recorded in Kattapana (41.00 per cent)and lowest incidence was recorded from Nedumkandam (8.33per cent) (Fig.1).

Banana bract mosaic virus, causing chlorotic mottle disease in small cardamom, which is associated with banana was for the first time reported in small cardamom by Siljo (2012)from different cardamom plantations in Kerala, Karnataka and Tamil Nadu during a survey for cardamom viral diseases. Chlorotic mottle disease incidence up to 15 per cent was recorded by Siljo (2012).

Many of the earlier researchers had reported the incidence of katte disease in small cardamom, while BBrMV was of recent occurrence. In the present study, both katte caused by CdMV and chlorotic mottle disease caused by BBrMV from small cardamom plantations cultivated in major blocks of Idukki district were observed in a moderate to severe form. The use of the disease affected clumps for propagation has resulted in the spread of the disease in major cardamom growing taluks of the Idukki district.

Banana aphid (*Pentalonia nigronervosa*) had been reported to be the vector of both the viruses (Uppal et al.,1945; Varma and Kapoor, 1958; Biju and Siljo,2012). Breeding of *P.nigronervosa f.sp.caladii* on small cardamom, Colocasia and caladium had been reported (Siddapaji and Reddy,1972; Venugopal,1995). In this study, the incidence of aphids as well as other hosts showing symptoms of viral infection as well as harbouring aphids was surveyed. No aphids were observed in the leaf sheath of cardamom as well as on other plants found near the plantations. The absence of aphids could be due to the frequent spraying of insecticides by the farmers against pests of small cardamom.

5.1.2 Maintenance of the virus inoculum

The virus infected plants were maintained at Cardamom Research Station, Pampadumpara for symptom development and aphid colonization. The plants were maintained under the shade conditions for further research work. It is essential to

maintain the virus infected small cardamom plants under shaded condition in order to perpetuate the viruses and the expressions of symptoms. Arya (2016).

5.2 SYMPTOMATOLOGY

Symptomatology of both the virus diseases affecting cardamom was studied. Katte disease affected plants were characterised by the presence of alternating light and dark green bands starting from midrib to the margin of the leaf. Severely infected plants produced stunted tillers. On the pseudostem, mosaic patches with white colour, extended white colour mosaic pattern and green colour mosaic patches were observed.

Plants with chlorotic streak disease expressed the presence of chlorotic flecks in spindle shape on the leaf sheath. In pseudostem symptoms like discontinuous running of spindle shaped streaks were observed.

Katte disease was also known as marble disease (Buchanan, 1807), as the first visible symptom was the presence of pale green discontinuous lines, and mosaic pattern in the form of mottling in the pseudo stem and leaves. Capoor (1967) had reported reduced size of leaf and pseudostem in katte disease as a result of CdMV infection.

Rao and Naidu (1973) had reported stunted growth and mosaic patterns on the pseudostem. Rao (1977) had differentiated the infected plants into three types A, B, C based on symptom development. Plants categorized under 'A type' showed dark longitudinal stripes, B type showed broken dark green bands with chlorotic stripes and C type showed stunted growth with granular mosaic pattern on the leaf portion. Such similar symptoms had been observed from the infected samples collected from different panchayats. Dimitman *et al.* (1984) had reported the presence of new shoots with greenish and yellow mottling on pseudostem and stunting of tillers. Gonsalves *et al.* (1985) had observed enlarged mosaic patterns on leaves during the collection of the infected samples of small cardamom. Venugopal (1995) reported severe stunting and reduced tillers production in case of severe infection of the virus diseases. In this study, the collected katte plants with severe infection expressed stunting of tillers as well as reduced tiller production.

In the case of chlorotic streak disease caused by BBrMV in small cardamom, Siljo *et al.*(2012)for the first time reported the incidence of the disease in the

100

cardamom plantations in South India. The commonly seen symptoms were continuous or discontinuous spindle shaped intra-veinal streaks along midrib and midvein regions of the leaf. These streaks later coalesce with each other and imparts yellow or light green colour to the veins. The chlorotic streaks were spindle shaped and were found as discontinuous streaks within the midrib region and margin region with interveinal chlorosis (Biju, 2012). In advanced stages, the pale green stripes are distributed evenly over the leaf surface giving a distinct mosaic pattern (Tiwari *et al.* 2016). Symptoms like intraveinal streaks, mottling of pseudo stem and petiole, loosening of leaf sheath and stunting of the plants were also reported (Bhat *et al.* 2018).

BBrMV was first detected in banana plantations during 1966 by Samraj *et al.* (1966) in Nendran variety in Thrissur district of Kerala. The yield loss was up to 40per cent (Rodoni*et al.*1997). Symptoms like discontinuous yellow colour stripes were found on the leaves. Some BBrMV affecting cardamom plants produced mosaic patches (Biju, 2010). Siljo (2012) reported that Kerala isolates of BBrMV produced light/ dark green mosaic patches along the midvein region in small cardamom. Bhat *et al.* (2018) reported that the cardamom plants belonging to Njallani (Green Gold variety)with severe infection had decreased tillers production, and expressed intravenous streaks, mottling of pseudo stem and petiole, loosening of leaf sheath and stunting of the plants. All the symptoms noticed by different researchers were noticed during our study also.

5.3SEROLOGICAL DIAGNOSIS AND MOLECULAR CHARACTERIZATION OF THE VIRUSES INFECTING CARDAMOM

5.3.1 DAC-ELISA - Direct Antigen Coated-Enzyme Linked Immunosorbent Assay

The samples collected from Kattapana, Pampadumpara and Nedumkandam were subjected to both DAC- ELISA and DAS-ELISA by using *Potato virus Y* (PVY) polyclonal antiserum at a dilution of 1: 500 and 1: 1000 dilution rate, the absorbance value for the infected sample was 0.655 while in case of healthy it was 0.226 for samples from Kattapana. The absorbance value for the infected sample was 1.388 while healthy was 0.226 from Pampadumpara and from Nedumkandam the absorbance value for the infected sample was 0.990 and healthy was 0.377. The

absorbance value for the infected samples from Ambalapara panchayat was 0.654 and for healthy it was0.179.The infected samples from Thukkupalam panchayat recorded an absorbance value of 0.908 and for healthy as 0.336; and in Chembalam panchayat the absorbance value for the infected sample was recorded as 1.388 and for healthy as 0.531. In all the infected samples, there was more than two-fold increase in OD value compared to the healthy control; thus, confirms the presence of the virus in the infected samples collected from different locations.

For the detection of chlorotic streak disease, DAC ELISA was done by using *Banana bract mosaic virus* (BBrMV) polyclonal antiserum at the primary antibody dilution of 1:200 and secondary antibody dilution of 1:10000. The absorbance value for the infected sample from Kattapana was recorded as 1.094 and healthy as 0.392, the absorbance value for the infected sample from Pampadumpara was recorded as 0.910 while healthy as 0.408.The absorbance value for the infected sample from Nedumkandam was recorded as 0.902 while that of healthy was 0.408.Thus, using ELISA with specific polyclonal antibody, it was possible to detect both the viruses.

Jacob *et al.*(2003) performed ELISA for detection of CdMV in infected samples collected from Yeslur, Appangala, Madikeri, Thalathamane, Sirsi, Kattappana, Vandiperiyar, Kurusupara and Palakkadusing CdMV IgG raised against Yeslur CdMV isolate with dilutions of 1:16000 and 1: 32000, and observed that Yeslur isolates recorded highest OD value compared to other isolates collected from different regions.

CdMV was detected at the dilutions of 1:1000 antigen dilution with 1: 4000 and 1: 10000 dilutions of polyclonal antibodies raised in mice and goat antimouse IgG alkaline phosphates conjugate (Smitha, 2004).Biju (2012) had performed ELISA for the detection of *Cardamom mosaic virus* by using three different dilutions of antisera at 1: 250, 1: 500 and 1: 1000 respectively and obtained positive results. Work done by Arya (2016)also revealed that the *Cardamom mosaic virus* was detected by using polyclonal antibody raised against katte mosaic virus in rabbit under the dilution ratios like 1:10, 1:100, 1:150 and secondary antibody in the ratio of 1:200.

Phadnis (2015) reported ELISA for detecting CdMV from Karnataka isolates, both indirect and direct ELISA were found to be helpful in detection. In case

of direct ELISA, polyclonal antibody specific to CdMV was used. Primary and secondary antibody dilutions used were 1:500, 1:1000 and secondary antibody dilution at 1: 1000. For indirect ELISA primary and secondary antibody dilutions at 1: 1000 were used.

ELISA was done to detect the viruses that cause katte disease by using PVY polyclonal antibody and chlorotic streak disease by BBrMV polyclonal antibody. From all the works done with ELISA, it could be concluded that both the viral diseases in small cardamom could be detected with varying dilutions / concentrations of primary and secondary antibodies. The reason may be the polyclonal type of antiserum which could bind at multiple sites of the antigen even at low concentrations of dilutions of antibody; thus, helping to detect the viruses.

5.3.2 Dot Immunobinding assay

DIBA was done as a part of method of detection using PVY and BBrMV polyclonal antisera at the dilution of 1:200 and 1:10000 for BBrMV whereas for the PVY the dilution ratio is 1:500 and 1:1000 as directed by the protocol of DSMZ. The NCM developed dark purple colouration for the infected samples and was colourless or less colour intensity in the case of healthy samples or with blank. Smitha (2004) performed DIBA for the detection of CdMV with antibody dilutions at 1:1000 and detected the presence of virus.

DIBA was performed to detect the presence of *Large cardamom chirke virus*(LCCV) in large cardamom by using polyclonal antibody (PAb) produced against the recombinant coat protein with a dilution of 1:5000 (Vijayanandraj *et al.*2013).Similarly, this method was used by Phadnis (2016), at 1:1000 for primary and secondary antibodies. Tiwari *et al.* (2016) detected CdMV in the infected samples at the dilutions of 1:10, 1:100 and 1:500 for antigen. Polyclonal antibody specific to CdMV at dilutions of1:500 and 1:1000for both primary and secondary antibodies were used.

5.3.3 Electron microscopy

Electron micrograph of katte disease infected samples revealed the presence of flexuous rodshaped particles of size 700 nm. Naidu *et al.* (1981; 1984) studied the

morphology of CdMV by electron microscopy and observed the flexuous filamentous virus particles with 650nm in length and 11-12 nm in breadth. Smitha (2004) reported a similar result of CdMV particles in the electron microscopy study. Dimitman *et al.* (1984) and Gonsalves *et al.* (1986) also observed the structure of CdMV as flexuous filamentous particles showing helix symmetry. Jacob and Usha (2001) reported that cardamom leaves infected with CdMV has shown filamentous flexuous particles with ~800 nm length and 11-12 nm breadth.

Tiwari *et al.* (2016) reported the presence of *Cardamom mosaic virus* with the help of electron microscopy where the flexuous rod shaped particles of 650 nm were observed. CdMV belongs to the family Potyviridae and is characterized by the presence of infective virions of size 650-900 nm. The result of electron micrograph is in consensus with other reports that CdMV is a flexuous rod virus.

5.3.4 Molecular Diagnosis

RT-PCR detection based on nucleic acid was done by isolating RNA, which was converted to cDNA and amplified using coat protein specific primers. The bands for three cardamom isolates from Kattapana, Pampadumpara and Nedumkandam for CdMV were amplified at 889 bp, 879 bp and 905 bp respectively. The bands for BBrMV from Kattapana, Pampadumpara and Nedumkandam were obtained at 625bp, 626bp and 633 bp respectively.

RT-PCR was done for *Cowpea aphid borne mosaic virus* which belongs to potyviridae, by Gillapsie (2001) and amplicons was observed at 221 bp. Jacob and Usha (2001) has reported 1.8 kb product containing the partial nuclear inclusion body (NIB) gene, the entire coat protein gene and 3' untranslated region (UTR) using RT-PCR.RT-PCR was performed with BBrMV specific primers and an amplicon of 950 bp was obtained for all the samples collected from different geographical region of Karnataka, Kerala and Tamil Nadu,

Phadnis (2015) had reported a 773 bp amplicon specific to CP gene using RT-PCR based detection of CdMV from samples collected from Karnataka. Arya (2016) obtained an amplicon of size 1000 bp, 750 bp and 650 bp with varying primers for coat protein region. In the present study an amplicon of size ranging from

889 -905 bp was obtained for CdMV infected samples whereas amplicon of size 625bp – 633bpwas obtained from BBrMV. The molecular size of the amplicons depends on the primers used for amplification. Many workers have reported the detection of RNA virus using RT-PCR which is found to one of the reliable methods for detection of these viruses.

5.3.4.1*Sequencing and BLAST Analysis*

On BLAST analysis it was observed that CdMV infected samples from Kattapana, Pampadumpara and Nedumkandam had shown similarity of 96.04per cent with *Indian Cardamom mosaic virus* (ICdMV) isolate of Thalathamane and 96.90per cent with CdMV isolates from Appangala. Whereas BBrMV infected samples from three panchayats has shown 91.01 per cent similarity with *Banana bract mosaic virus* CdM (Karnataka),90.29 per cent similarity with *Banana bract mosaic virus* isolate I1 polyprotein (Coimbatore),95.76 per cent similarity with *Banana bract mosaic virus* isolate KAPP2 (Thrissur),

Jacob and Usha (2001) confirmed that CdMV belongs to Macluravirus group by constructing a phylogeny tree. Jacob *et al.*(2003) reported that there was 42.5 per cent identity in CP region for Vandiperiyar and Thalathamane region, while there was 67.5 per cent identity for Kurusupara and Kattappana isolates infected with CdMV. Phadnis (2015) reported that CdMV from Sakleshpur had shown 54.51 per cent similarity with *Cucumber vein yellowing virus* (CVYV) (Ipomovirus), 49.59 per cent with *Cardamommosaic virus* (CdMV) (Macluravirus), whereas Mudigere isolate has shown similarity of 96.50 per cent with *Sugarcane mosaic virus* (SCMV) (Potyvirus), 90.78 per cent with *Potato virus Y* (PVY) (Potyvirus), 60.36 per cent with *Alpinia mosaic virus* (AlpMV) (Macluravirus).

The phylogeny tree for CdMV isolates from Kerala and Karnataka recorded 87.1- 98.9 per cent similarity for all Kerala isolates which clustered together with Tamil Nadu isolates (Bhat *et al.* 2018). The reason for similarity between viruses may be due to continuous cultivation of the infected clumps.

5.4 ELIMINATION OF VIRUS THROUGH MERISTEM TIP CULTURE

Rhizomes from infected samples were collected and inoculated into the MS medium supplemented with coconut water, 2mgL^{-1} BAP and 1 mgL^{-} IAA for shoot initiation. The rooting medium containing MS medium supplemented with coconut water, 3.5 mgL^{-1}of IAA and 1.5 mgL^{-1}of BAP resulted in rooting. The regenerated plants were obtained within 3 months and grown normally without intervention of callus growth. The plants were subjected to the ELISA and RT-PCR for virus indexing. The results for ELISA were negative and no sample had been detected with the presence of virus. RT-PCR method confirmed that meristems were free from both the viruses.

Srinivasa *et al.* (1982) raised plantlets by using seeds and inoculated them into the MS medium supplemented with 2 per cent sucrose, 2, 4-,D(2mgL^{-1}),Indole-3-butyrie acid (2mgL^{-1}) and naphthalene acetic acid (2mgL^{-1}) and benzyl amino purine (BAP - 2mgL^{-1}). Nadaguda *et al.* (1983) also reported raising plantlets by using young buds where MS medium was supplemented with NAA 1 mgL^{-1}, IAA 1.5 mgL^{-1}, Kinetin 0.5 mgL^{-1}, coconut water 5 per cent, calcium pantothenate (0.1 mgL^{-1}) and biotin (0.1 mgL^{-1}).

Smitha (2004) has produced virus free plantlets in MS medium supplemented with Kinetin 0.2mgL^{-1}+ BAP 1.0 mgL^{-1} + IBA 0.2mgL^{-1}and NAA 0.5mgL^{-1} and ribavirin to inhibit culture contamination and subjected to virus indexing for cardamom plants and found that all the meristems were free from virus. Tyagi *et al.* (2009) raised cardamom plants under slow conservative growth method by using ½ MS medium supplemented with5 mgL^{-1} BAP, 3 per cent sugar and 0.7 per cent agar with callus phase within 18 months.

It could be concluded that the virus could be eliminated by meristem culture and plants could be raised with both direct and indirect organogenesis mechanism. Cytokinins were more effective in shoot initiation as Kinetin, BAP and coconut water contains cytokinin hormones. But more studies are required for the standardization of the conditions for getting indirect organogenesis for the production of a greater number of plants from a single meristem.

ELISA with PVY polyclonal antiserum for detection of CdMV (Smitha, 2004) and BBrMV polyclonal antibody for the detection of BBrMV infection was undertaken to confirm the absence of virus in the multiplying shoot. RT-PCR with primers specific for coat protein region was employed to confirm the absence of CdMV and BBrMV virus (Biju, 2012).

Thus, this study revealed the presence of two major viruses viz., CdMV and BBrMV in major small cardamom cultivated blocks of Idukki district through serological and molecular detection. More studies need to be conducted to standardize the economically feasible production technique of virus free plants from meristem.

Summary

6. SUMMARY

The present study deals with characterization of different viruses infecting small cardamom and the production of disease free plants. The study was carried out in the Department of Plant Pathology, College of Agriculture, Vellayani during the period from 2017-2019.

Survey was conducted in two major cardamom cultivating blocks from Idukki district. A total of six panchayats from Kattappana and Nedumkandam blocks were surveyed for the incidence of the viral diseases. The two viral diseases prevalent were katte disease and chlorotic streak disease. The katte incidence recorded from small cardamom plantations in Kattappana was 28.20 per cent, in Pampadumpara was 43.00 per cent, Nedumkandam was 31.25 per cent, Thukkupalam was 8.33 per cent, Ambalapara was 3.75 per cent, and Chembalam was 5.00 per cent. The incidence of chlorotic streak from Kattappana was 41.00 per cent, Pampadumpara was 30.00 per cent, and Nedumkandam was 8.33 per cent. Highest disease incidence of katte disease was recorded from Pampadumpara (43.00%) and the lowest from Ambalapara panchayats (3.75%) while highest disease incidence of chlorotic streak disease was noted from Kattappana (43.00%) and the lowest from Nedumkandam (8.33%).

Symptomatology studies were conducted for both katte and chlorotic streak diseases. Katte infected plants showed mosaic patterns on their leaves and pseudostem. The distinct feature of the disease was the presence of discontinuous parallel lines with light and dark green colour alternating each other that extends from midvein region to leaf margins. The infected leaves expressed variations in mosaic patterns from the different panchayats. The pseudostem expressed whitish mosaic pattern during early stages of infection and at later stage, symptoms like enlarged green colour mosaic patterns were also observed. Severe infection of the clumps was characterised by stunted growth with reduced tillers. The plant remained short and resulted in reduced yield.

Chlorotic streak disease infected leaves expressed interveinal chlorosis. The infected pseudostem showed the presence of discontinuous spindle shaped

marks while leaves had small chlorotic fleck that was yellowish in colour along the vein and it extended from midvein region to margin region.

Serological techniques like DAC-ELISA, DAS-ELISA and DIBA were done to detect the presence of viruses. DAS-ELISA for the detection of *Cardamom mosaic virus* (CdMV) causing katte disease was done by using polyclonal antibody of PVY (DSMZ Pvt. Ltd). The dilutions of primary and secondary antibody used were 1:500and 1: 1000 respectively. In the case of detection of chlorotic streak disease, polyclonal BBrMV antibody (DSMZ Pvt. Ltd) was used in DAC-ELISA. The dilution of primary antibody was 1:200 while the secondary conjugated antibody was used at 1:10000. All the diseased samples collected from different panchayat gave positive reaction in ELISA. Highest OD value was recorded for katte disease from Pampadumpara (1.388), and for chlorotic streak disease, the highest OD value (1.079) was recorded in the samples from Kattappana panchayat.

DIBA was also performed as a serological tool to confirm the presence of CdMV and BBrMV in the infected samples. All the samples that were tested has given the positive result with good purple colour development on the disease samples spotted regions. Polyclonal antibody of PVY primary at a dilution at 1:500 and secondary conjugated antibody at 1:1000 dilution was used for detection of CdMV while polyclonal antibody of BBrMV at a dilution of 1:200, and secondary antibody at a dilution of 1:10000 used for detection of BBrMV causing chlorotic streak disease in small cardamom.

Molecular diagnosis was performed by using coat region specific primers for the amplification. As both the diseases are caused by RNA viruses, RNA was isolated, reverse transcribed and PCR was done to amplify the coat protein region. Amplicons obtained for CdMV samples from Kattappana, Pampadumpara and Nedumkandam panchayats were of the size 879, 889 and 905bp respectively. The BBrMV samples produced amplicons of size 625, 626 and 633 bp for Kattappana, Pampadumpara and Nedumkandam panchayats samples respectively.

Phylogeny tree was constructed for CdMV and BBrMV infected isolates from Kattappana, Pampadumpara and Nedumkandam samples and compared with CdMV and BBrMV isolated from different small cardamom grown regions in India listed in NCBI site. CdMV Kattappana and Nedumkandam isolates were clustered together and CdMV Pampadumpara was in another clade. BBrMV Pampadumpara and Nedumkandam were clustered together, while BBrMV Kattappana was in a separate clade.

The sequences of virus causing katte disease in Kattappana subjected to BLAST analysis was found to be 96.04 per cent similar to *Indian cardamom mosaic virus* from Thalathamane, while the virus isolate from Pampadumpara was also found to be 97.33 per cent similar to *Indian cardamom mosaic virus* isolate from Thalathamane and Appangala. The *Cardamom mosaic virus* (CdMV) from Nedumkandam was closely related to CdMV isolate from Appangala with 96.90 per cent similarity. The virus associated with chlorotic streak in cardamom from Kattappana was related to *Banana bract mosaic virus*(BBrMV) CdM isolate Karnataka with 91.01 per cent similarity, while BBrMV from Pampadumpara was related to *Banana bract mosaic virus* isolate I1 polyprotein Coimbatore with 90.29 per cent similarity and BBrMV from Nedumkandam was related to BBrMV isolate KAPP2 polyprotein Thrissur with 95.76 per cent similarity.

Meristem culture was done to eliminate the virus present in the samples collected from mother plants that were infected with both CdMV and BBrMV from surveyed locations. Meristems from infected rhizomes were isolated and placed on to the MS medium supplemented with $3mgL^{-1}$ BAP, 1.5 mgL^{-1} IAA and 0.8 mgL^{-1} Kinetin in order to produce plantlets through direct organogenesis. Proliferated plantlets were subjected to serological and molecular diagnosis which indicated the absence of viruses in the meristem.

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Appendices

APPENDIX I

Buffers for DAC-ELISA and DAS-ELISA

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

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

2. **Wash buffer (PBS-T)**

Add 250µL of Tween 20 to 500 ml of PBS

3. **Coating buffer(pH 9.6)**

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

4. **Antibody diluents buffer**

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

5. **Enzyme conjugate diluents buffer**

Same as PBS-TPO

6. **Substrate solution (pH 9.8)**

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

APPENDIX II

Buffers for DIBA

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

   0.02 M Tris - 4.84 g

   0.5 M NaCl -58.48 g

   Adjust pH to 7.5 with 1N HCl and make up to 2 litres, this can be used as washing solution.

2) **Antigen extracting buffer (TBS-SDM)**

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

3) **Blocking Solution**

   Spray dried milk 5 g

   TBS buffer 100 ml

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

4) **Antibody and enzyme- conjugate diluent/ buffer**

   Add antibody of required diluents to TBS-SDM

5) **Substrate buffer (pH 9.5)**

   0.1 M Tris 12.11g

   0.1 M NaCl 5.85 g

   5 mM $MgCl_2$. $6H_2O$ 1.01 g

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

6) **Substrate solution**

   Solution A

   Nitro Blue tetrazolium (NBT)-75 mg

   Dimethyl formamide (DMF) -1 ml

   Solution B

   Bromo chloro indolyl phosphate (BCIP) - 50 mg

   Dimethyl formamide (DMF) -1 ml

7) **Fixing solution (pH 7.5)**

10mM Tris	1.21 g
1mM EDTA	0.29 g

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

APPENDIX III

RNA isolation

Chemicals and solutions

Trizol reagent	-5 µl
βmercaptoethanol	-5 µl
sodium sulphite	-50 µl
sodium acetate	-500 µl
Saturated phenol	-500 µl
Chloroform	-100 µl
Isopropanol (ice cold)	-500 µl
Ethanol (75%)	-200 µl
Nuclease free water	-50 µl

Requirements for cDNA synthesis

cDNA synthesis buffer	-4 µl
dNTP mix	-2 µl
RNA primer	-1 µl
RT enhancer	-1 µl
Verso enzyme mix	-1 µl
RNA	-5 µl
Nuclease free water	-6 µl

Requirements for RT-PCR synthesis

10X Polymerase buffer	-5 µl
0.1M DTT	-5 µl
dNTPs	-2.5 µl
DNA Polymerase	-3 µl
Enzyme mix	-1.5 µl
Forward primer	-1 µl
Reverse primer	-1 µl

Template	-15 µl
Nuclease free water	-16 µl

APPENDIX IV

Stock solutions for tissue culture

Stocks solutions	For 1 litre	For ¼ litre	Full MS	Half MS
STOCK 1				
NH_4NO_3	82.5 g	20.625g	20 ml	10 ml
KNO_3	95 g	23.75 g		
KH_2PO_4	8.5g	2.125 g		
$MgSO_4.7H_2O$	18.5 g	4.425 g		
STOCK 2				
$CaCl.2H_2O$	22 g	5.5 g	20 ml	10 ml
STOCK 3				
Na_2 EDTA	3.7 g	0.925 g	10 ml	5 ml
$FeSO_4.7H_2O$	2.8 g	0.7 g		
STOCK 4				
$MnSO_4.4H_2O$	2.23 g	0.5575 g	10 ml	5 ml
$ZnSO_4.7H_2O$	0.86 g	0.215 g		
H_2BO_3	0.62 g	0.155 g		
KI	0.083 g	0.02075 g		
Na_2MOO_4	0.025 g	0.00625 g		
$CuSO_4.5H_2O$	0.0025 g	0.000625 g		
$CoCl_2.6H_2O$	0.0025 g	0.000625 g		
STOCK 5				
Glycine	0.2 g	0.05 g	20 ml	10 ml
Nicotinic acid	0.05 g	0.0125 g		
Pyridoxine acid HCl	0.05 g	0.0125 g		
Thiamine HCl	0.01 g	0.0025 g		
Ingredients	**Full MS**		**Half MS**	
Inositol	0.1 g		0.05 g	

Sucrose	30 g	15 g
Agar	8.0 g	4.0 g
Coconut water	100 ml	50 ml

Hormones	**Weight in milli grams to be dissolved in 100 ml distilled water**
Benzyl aminopurine (BAP)	1
Indole acetic acid (IAA)	1
Kinetin (Kn)	1

*Dissolve$FeSO_4.7H_2O$ and Na_2. EDTA separately, store them in a dark place or amber bottles. Later mix them and make up to the stock required.

After adding all stock solutions and hormones, inositol, agar and sucrose were added and volume was made up to 750 ml. pH was adjusted to 5.8 and made up to 1 litre.

APPENDIX V

CdMV isolate from Kattappana Panchayat

TGGGGGGACAGCGCACAGAAAGAGACATCTAATTATATAAAATGAGT
GCATATTTGTAAACACACTAAATTGACATACCGATATGTTCAATATAG
AGTTGCTCGTGGCTCACCTATTGAGCCATGTTCAAATTCGTCGACATCA
GTGTCGACATGCCTCTCATAACTCGTCTTGCTTCGCTGGACTTTCCCGT
CTAGTAGCATCACTCGCTGGTAACCTGAACCAATTGCAGCAGCTTTTC
CTTGATTTAATTGCTCGCGCACTGTTTTGGGCATCCCATTCGTTTGAAT
GCAAAAATCAAAAGCGTATGGTATCATTGCAAGCTGTGTGTAACCTCT
CTTTGTTCCCCATGCCGTCATTTTTCCTCCTTTGGCTAGTATTTGTGATG
TAATATCACTGAGATGTCTCATAATTTTCCGAAGCCCTCCGTTTTGCAC
TGCTGGTTCGACAAAGATGGCAATTGGTATTGTGGAGTACTTCCCACC
TGAGTGAATTTCCATGTTTTGGTTGACGTCAACTTCTGAAGAGGTTCCG
TTGTTGGCACACCACAAACACCATGATGTTAAATAAATTTGAAAGTCA
TCTTCATTTGGTGTTCCAAGACTCTTCCTAACATCTTCAGCCCATCTCTC
AAACTGCAAAGTTGTTGCTAATTGTGATGATTGTGTGAACTGTTCCTTT
GCGATGCTGTTAATTATCTTTCTGTTCCAAATCCTTTTACCTTTCACGCG
TGGGTTGTTGAAGTGACTCAACCTCCTTGGTGTTGGGGGTATTCTCCAC
TGAATGTCCGTGTCGTCATCGCGCGCTAGTGCAAATCTTGTGCTGAGC
ATCAAAGCTCTCAACCGGTGCGTGGTTTCCGCATGTGCTGGCTGTCAT
GCG

APPENDIX VI

CdMV isolate from Pampadumpara Panchayat

TGGCATTGGACTGTCCACAGATAGAGACATCTAATTATATAAAATGAG
TGCATATTTGTAAACACACTAAATTGACATACCGATATGTTCAATATA
GAGTTGCTCGTGGCTCACCTATTGAGCCATGTTCAAATTCGTCGACATC
AGTGTCGACATGCCTCTCATAACTCGTCTTGCTTCGCTGGACTTTCCCG
TCTAGTAGCATCACTCGCTGGTAACCTGAACCAATTGCAGCAGCTTTTC
CTTGATTTAATTGCTCGCGCACTGTTTTGGGCATCCCATTCGTTTGAAT
GCAAAAATCAAAAGCGTATGGTATCATTGCAAGCTGTGTGTAACCTCT
CTTTGTTCCCCATGCCGTCATTTTTCCTCCTTTGGCTAGTATTTGTGATG
TAATATCACTGAGATGTCTCATAATTTTCCGAAGCCCTCCGTTTTGCAC
TGCTGGTTCGACAAAGATGGCAATTGGTATTGTGGAGTACTTCCCACC
TGAGTGAATTTCCATGTTTTGGTTGACGTCAACTTCTGAAGAGGTTCCG
TTGTTGGCACACCACAAACACCATGATGTTAAATAAATTTGAAAGTCA
TCTTCATTTGGTGTTCCAAGACTCTTCCTAACATCTTCAGCCCATCTCTC
AAACTGCAAAGTTGTTGCTAATTGTGATGATTGTGTGAACTGTTCCTTT
GCGATGCTGTTAATTATCTTTCTGTTCCAAATCCTTTTACCTTTCACGCG
TGGTTGTTGAAGTGACTCAACCTCCTTGGTGTTGGGGGTATTCTCCACT
GAATGTCCGTGTCGTCATCGCGCCGCTAGGTGCAAAATCTTGTTGCTG
AGCATCAAAGCTCTCAACCGGTTGCGTGGGTTTTCCGCATGTTGCTGG
CCTGTCATGCG

APPENDIX VII

CdMV isolate from Nedumkandam panchayat

CAAGCGTATTGATCGTTGACCACACCGGCTACAAGGCCTGTACGCTCAATTGC
AAGAATCAACGCTTGGTGGTGGCGATGATGACGCAGACGTTCTCTGGAGGAT
ACCACCAACACCGATAAGGCAGAGGCACTTTATGAACCCACGAGTCATGGGA
AAGAGAATCTGGAATAGGAAAATTATCAACAGCATTGCAAAAGAGCAATTCA
CACAATCATCACAGTTGGCAACCACCTTGCAATTTGAAAAATGGGCTGAGGA
TGTTCGGAAAAGTCTCGGTACACCCAATGAAGAAGATTTTCAAATTTATCTCA
CATCATGGTGTTTATGGTGTGCCAATAATGGCACATCATCAGAGGTTGATGTC
AACCAGAACATGGAGATTCATTCAGGTGGGAAGTACTCGACAATACCAATTG
CCATCTTTGTTGAGCCAGCAGTGCAAAATGGCGGGCTTCGGAAGATCATGAG
ACACCTCAGCGATATAACATCACAAATATTAGCCAAAGGTGGAAAAATGACA
GCATGGGGAACGAAGAGGGGTTACACGCAACTCGCAATGATACCTTATGCTT
TTGATTTTTGTGTTCAGACAAATGGGATGCCCAAAACAGTTCGTGAGCAGCTA
AATCAAGGGAAAGCTGCTGCAATTGGTTCAGGCTACCAACGAGTCATGCTTC
TGGATGGGAAGGTGCAACGCAGCAAGACGAGTTATGAAAGGCATGTCGACA
CTGATGTTGACGAATTTGAACACGGCTCCACGAGTGAGCCACGAGCAACCCT
ATATTGAACATATGTTGATGTCAATTTAGTGTGTTTTAAAGTTCATTTTAATAA
ATGTTTAATAATCACTACTTGCTGCATCTATAGGTTCAATGGGAGTTTGTTGG
GGGGTTTCCACA

APPENDIX VIII

BBrMV isolate from Kattappana panchayat

TAGTCGCTCTGTCACTTGTTGCCTACGGGGAGGATTAGTTTACATTTTA
GCCTTTCTTGATTTGTCCACCCTTCTTACCGATCATTATGCCAATTTTTT
CGATGAAAGTAAGGCCTACTTCACAATGCACGTGTCACGGAAAAATAT
TACCCAGGTGCGCAGCACTTAGAGGATTGATGAATAGCTTAGCCCGAT
ATGCATTTGATTTTTACGTAATGCATCAAAACTACCACAGGGCTAGAG
AAGCTCACACGCGGATTATGCTGCAGCTATTCGTGGATCAAAACTCGG
TTATTCGGTTTGGATGGAAATCTTGGACCCGGTGAAGAGAATACAGAG
AGGCATACTGTTGAAGATGTGAAGCGTGATATGCACTCTCTGCTTGGG
ATGAAACATGAAAAAATAAATAGTCATCTGGAGCTTGCTCCTTATAAC
TATGTGTGCTTTATGATATTGTGATAATTGTAGTGTGAGCTTCTCACCT
AAAGTACCTACATGCATTGTGTGTGGTATTTATATATTCGCAATATGCG
AGAGGGACCGCGTTGTGAGGTATTGACCAAAGTGATAATGGTTATCCA
GAGTCTCTATCCTAGCTGGCGTGCACAACCTTCTAATAAAAATGA

APPENDIX IX

BBrMV isolate from Pampadumpara panchayat

TCCCGGCTCTCGTGCCTGCTTGATGCCTACGGGGAGGATTTCTTTACAT
GTTTAGCCTATTCTTGTATATGTTCACCTTTCTTTTCGATTATTATGCCT
CATTTTTCTAATGCTGCTGAGCCACACTTTGCAATGCGCATGTCACGTA
AAGATATTGCCCAGGTGCGTAGCACTTATAGGATTGAATGACATAGCT
TAGCCCGATATGCATTTGATTTTTACGTAATCACATCAAAAACTACAA
ACAGGGCTAGAGAAGCACACACGCAGATCTAGCTGCAGCTATTCGTG
GATCAAACACTCGGTTATTCGGTTTGGATGGAAATCTTGGACCCGGTG
AAGAGATACAGAGAGGCAAACTGTTGAAGATGTGAAGCGTGCTATGC
ACTCTCTGCTTGGGATGAAACAGAATAAATAATAGACATCTGGAGGTG
CTCCTTATAACATGTGTGCTTTATGATATTGTGATAATTGTAGTGTGAG
CTTCTCACCTACGTACCAACATGTTTGGGGTGTGGATTTAAATATCCGC
ATATGAAAAGGGAGCGGCTGTGAGTATTGAGAAGTGATAATGGTTATC
AGGAGTCTTTAGTAGCTGGGGGGCACACCCTTAGAAAAAATGGGT

APPENDIX X

BBrMV isolate from Nedumkandam panchayat

GCCTCGGCTTCTGGTGTTGATGGATAGGGGAGCATTAGTTTACCATGTT
TAAGCCTATTCTTGAGAATGCTCACCCTACTTTTCGATAATTATGCCAC
ATTTTTCTGATGCTGCTAAGGCATACTTAACAATGCGCAATGTCACGG
AAAGATATATGCCCAGGTGGGGAGCACTTAGAGGATTGAATGACATA
AGCTTAGCCCGATATGCATTTGATTTTTACGTAATCACATCAAAAACTA
CAAACAGGGCTAGAGAAGCACACACGCAGATGAAAGCTGCAGCTATT
CGTGGATCAAACACTCGGTTATTCGGTTTGGATGGAAATCTTGGACCC
GGTGAAGAGAATACAGAGAGGCATACTGTTGAAGATGTGAAGCGTGA
TATGCACTCTCTGCTTGGGATGAAACATGAATAAATAAATAGTCATCT
GGAGCTTGCTCCTTATAACTATGTGTGCTTTATGATATTGTGATAATTG
TAGTGTGAGCTTCTCACCTAAGTACCTACATGTATTGTGTGTGGTATTT
ATATATTCGCAATATGCAAAGGGACCGCCTGTGAGGTATTGACCAAGT
GATAATGGTTATCCAGAGTCTCTATCTAGCTGGCGAGCACACATAGGG
AATATAT

Abstract

CHARACTERIZATION OF DIFFERENT VIRUSES INFECTING SMALL CARDAMOM (*Elettaria cardamomum* Maton) AND PRODUCTION OF DISEASE FREE PLANTS

by

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ABSTRACT

The study entitled "Characterization of viruses infecting small cardamom (*Elettaria cardamomum* Maton) and production of disease free plants" was conducted at Department of Plant Pathology, College of Agriculture, Vellayani during 2017-2019, with the objective to study the occurrence and distribution of viruses infecting small cardamom, molecular characterization of the viruses and elimination of viruses through meristem culture for the production of diseasefree planting material.

Survey was conducted in Kattappana, Pampadumpara and Ambalapara panchayats of Kattappana block; and Nedumkandam, Thukkupalam and Chembalam panchayats of Nedumkandam blocks of Idukki district during November 2018 – May 2019. The incidence of katte disease caused by *Cardamom mosaic virus* (CdMV) was present in all the panchayats surveyed and it ranged from 3.75 to 43.0 per cent in Kattappana block and 5.0 to 31.33 per cent in Nedumkandam block. Disease incidence for chlorotic streak caused by *Banana bract mosaic virus* (BBrMV) was recorded from Kattappana (41%), Pampadumpara (30%) and Nedumkandam (8.33%)panchayats. The aphids infestation was absent in all the surveyed plots. *Colocasia*spp. and *Alpinia* spp. were the major plants observed in and around the cardamom fields and were not having visible symptoms of the viral infections. The virus inoculums were maintained under insect proof net house at Cardamom Research Station, Pampadumpara.

Katte disease produced slender chlorotic flecks developing into pale green discontinuous stripes running parallel to veins from midrib to leaf margin of the infected leaves. Mosaic mottling and chlorotic specks were seen on the infected leaves and young pseudostems. In case of severe infection, plants produced stunted tillers. Chlorotic streak disease was characterised by continuous and discontinuous chlorotic streaks along veins and midribs of the infected leaves and green discontinuous spindle streaks on pseudostem.

CdMV(a potyvirus) and BBrMV in cardamom was detected using polyclonal antibodies of *Potato virus Y* (PVY) and BBrMV respectively procured from DSMZ, Germany by direct antigen coating- Enzyme linked immunosorbentassay (DAC-ELISA) and Dot immunobinding assay (DIBA).The highest virus titre of CdMV and

BBrMVwas obtained in samples collected from Pampadumpara and Kattappana respectively. Molecular detection of the viruses was carried out using reverse transcriptase - polymerase chain reaction (RT-PCR) with specific primers for CdMV and BBrMV; and obtained amplicons of expected size of 879-905 bp for CdMV- and 625-633 bp for BBrMV- infected samples. The sequences of the isolates of CdMV from Kattappana, Pampadumpara and Nedumkandam were subjected to BLAST analysis and found to be similar to *Indian cardamom mosaic virus*isolates from Thalathamane and Appangala with > 96 per cent similarity. The BBrMVin cardamom from Kattappana, Pampadumpara and Nedumkandam was similar to *Banana bract mosaic virus* (BBrMV) CdM isolate of Karnataka (91.01%),Coimbatore (90.29%) and Thrissur(95.76%) respectively.

Phylogeny tree constructed in MEGA 6.0 software differentiated CdMV and BBrMV into four clades, in which CdMV Kattappana and Nedumkandam isolates were clustered together whereas CdMV Pampadumpara isolate was in separate clade. Similarly, BBrMVisolates of Pampadumpara and Nedumkandam clustered together while BBrMVKattappana was in separate clade.

Meristem of 2mm size separated from infected plants were grown in Murashigeand Skoog medium supplemented with 3mg benzyl amino purine (BAP), 1.5 mg indole acetic acid (IAA) and 0.8 mg kinetin expressed direct organogenesis but multiple shoots were not produced. The TC plants were subjected to DAC-ELISA with the specific polyclonal antibodies and PCR with specific primers of the viruses and confirmed that the plants produced from meristems were free of both the viruses.

Thus, the present studyrevealed that two viral diseases *viz*., katte and chlorotic streak affecting small cardamom in Idukki. Serologically and molecularly it was detected that katte disease was caused by *Cardamom mosaic virus* (CdMV) and chlorotic streak disease was caused by *Banana bract mosaic virus* (BBrMV), and the viruses could be eliminated from the infected plants through meristem tip culture to produce the diseases free plants.