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MOLECULAR CHARACTERIZATION OF KATTE MOSAIC VIRUS OF CARDAMOM (*Elettaria cardamomum* Manton)

By

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(2014-11-102)

THESIS

Submitted in partial fulfilment of the requirement
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CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY
COLLEGE OF HORTICULTURE
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KERALA, INDIA

2016

DECLARATION

I hereby declare that the thesis entitled **"Molecular characterization of *Katte mosaic virus* in cardamom (*Elettaria cardamomum* Manton)"** is a bonafide record of research work done by me during the course of research and that it has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

Vellanikkara

Date: 03-09-2016

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Certified that the thesis entitled **"Molecular characterization of *Katte mosaic virus* in cardamom (*Elettaria cardamomum* Manton)"** is a record of research work done independently by **Mr. Manglam Arya (2014-11-102)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship, fellowship to him.

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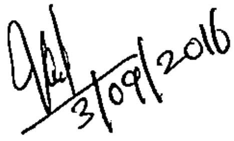

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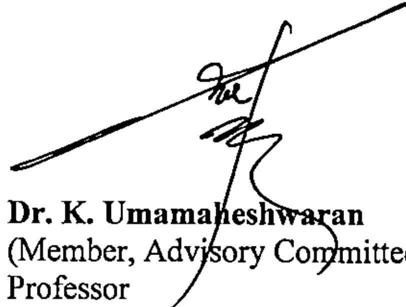

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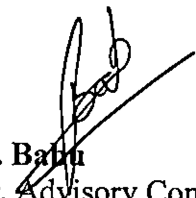

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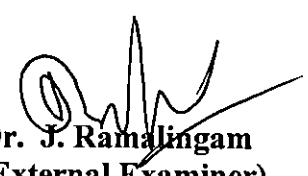

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ABBREVATIONS

APS	Ammonium per sulphate
APS	Alkaline phosphatase
B	Beta
bp	Base pair
BLAST	Basic local alignment search tool
BBrMV	*Banana bract mosaic virus*
CPBMB	Centre for Plant Biotechnology and Molecular Biology
CdMV	*Cardamom mosaic virus*
CTAB	Cetyl Tri-methyl Ammonium Bromide
C-DNA	Complementary Deoxyribonucleic Acid
CdMV	*Cardamom mosaic virus*
CMV	*Cucumber mosaic virus*
DNA	Deoxyribonucleic Acid
DEPC	Diethyl pyrocarbonate
DIC	Distributed Information Centre
DNase	Deoxyribonuclease
DAC- ELISA	Direct antigen coating Enzyme linked immunosorbent assay
DIBA	Dot immunobinding assay
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
G	Gram
ha	Hectare
HRP	Horse Radish Peroxidase
Ig	Immunoglobulins
Kb	Kilo basepairs

kDa	Kilo dalton
KP	Potassium phosphate
L	Liter
M	Molar
mM	Mili molar
mg	Mili gram
ml	Mili liter
MOPS	3-(*N*-morpholino) propane sulfonic acid
$MgCl_2$	Magnesium chloride
µg	Microgram
µl	Micro liter
ng	Nano gram
NIB	Nuclear inclusion body
°C	Degree Celsius
OD	Optical Density
ODD	Ouchterlony Double Diffusion
%	Percentage
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration
pM	Pico molar
PEG	Polyethylene glycol
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
pPNP	para-Nitro phenylphosphate
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
RNase	Ribonuclease
RT-PCR	Reverse transcriptase polymerase chain reaction

rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
TEMED	Tetramethyl ethylenediamin

Introduction

1. INTRODUCTION

Small cardamom (*Elettaria cardamomum* Maton) is a perennial bushy herbaceous monocotyledonous plant originated in the mountainous region of the South-Western part of the Indian peninsular and commonly referred to as "Queen of Spices". The genus *Elettaria* belongs to family Zingerberaceae having diploid chromosome number 2n=48. Cardamom is a shade loving crop cultivated under the shades of evergreen trees. It is mainly propagated through seeds, suckers, rhizomes and tissue culture plantlets. The flowers are hermaphrodite and emerge as long floral stalk directly from the underground stem. Cardamom start bearing fruits from third year after planting, and continue up to 8-10 year (Purseglove, 1981). In India, cardamom is mainly cultivated for its fruit and oil which is an important ingredient in food, medicines and beverages industries (Bhat and Nesamony, 1986). Guatemala is the leading producer and exporter of cardamom. Commercial producers of cardamom are Guatemala, India, Tanzania, Srilanka, Vietnam and Papua New Guinea. In India, cardamom is mainly cultivated on the hilly tracks of Kerala which contributes about 60 per cent of cultivation and production followed by Karnataka (30 per cent) and Tamil Nadu (10 per cent) (Madhusoodanan *et al.*, 1994).

Productivity of cardamom in India is very low (about 145 kg/ha) due to the wide spread of viral diseases affecting cardamom plantations. Unavailability of quality planting material is further enhancing the susceptibility of cardamom to viral infections as it is propagated by vegetative means. The Katte or mosaic disease is one of the most severe diseases affecting cardamom. The infected leaf shows chlorotic flecks later developing into dark green stripes that run parallel from the midrib to margin over the entire leaf surface and shows mosaic pattern when disease is fully developed (Uppal *et al.,* 1945).

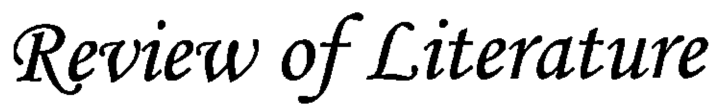
Disease was first reported by Thomas (1939) and then Uppal *et al.* (1945) confirmed the viral nature of the disease. Primarily, the virus is transmitted by use of

infected rhizome and transmittance by *Pentalonia nigronervosa* is secondary (Rao, 1977). *Katte mosaic virus* or *Cardamom mosaic virus* (CdMV) causal organism of Katte disease is a member of the family Potyviridae which can be seen as flexuous filamentous particles of about 650 nm in length and 10-12 nm in diameter and belongs to the genus *Macluravirus* (Jacob and Usha, 2001).

Common methods such as breeding for plant virus resistance and cross protection with mild strains show only limited success in control Katte disease (Gonsalves *et al.*, 1986 and Madhusoodanan *et al.*, 1990). The other methods such as chemical for pest control and cultural practices are not useful in managing the virus. Management of Katte disease is totally dependent on the use of disease free planting material such as rhizome and suckers but sometimes the virus remain as symptomless carrier or shows mild symptoms in the planting material so identification and diagnosis of virus becomes difficult. Hence, the disease remains as the major constraint in cardamom cultivation in India. Therefore, it is necessary to develop easy and early diagnostic technique for the detection of *Katte mosaic virus* to avoid the use of infected rhizomes or suckers as planting materials and also prevent the spread of the virus in other plantation of cardamom.

Based on the above information, the present study was undertaken with the following objective:

1. To develop serological and PCR based methods for identification and characterization of Purification of *Katte mosaic virus* of cardamom


Review of Literature

2. REVIEW OF LITERATURE

Small Cardamom *(Elettaria cardamomum* Maton.) also referred to as 'Queen of spices' is an important spice crop cultivated in India. It belongs to the family Zingerberaceae. Katte or mosaic disease of cardamom is a destructive viral disease caused by *Katte mosaic virus* or *Cardamom mosaic virus* (CdMV) that results in low productivity of cardamom in India. Lack of reliable diagnostic techniques for the identification of *Katte mosaic virus* leads to widespread of the disease. In the present investigation, we attempt to isolate the coat protein of virus and development of various diagnostic techniques such as ELISA and RT-PCR for the easy and early detection of the virus. Various reports on Katte disease, *Katte mosaic virus,* molecular diagnosis of viruses has been reviewed and presented in this chapter.

2.1. Disease Incidence and Crop Losses

The disease is prevalent throughout the cardamom growing countries including Srilanka, Guatemala and India. In India, the disease was first reported from South India in 1907 by Buchanan. Van Bundren (1914) believed that the disease was of vague nature and the plantations became unproductive within 3-4 years of infection. Mayne (1951) observed that Katte disease was widely distributed in the cardamom growing regions of South India (Karnataka, Kerala and Tamil Nadu) with about 0.01 to 99 per cent of reported incidence.

Varma and Capoor (1953) observed that there was loss in total yield of cardamom when the virus affected the cardamom plantations at early stage of growth. It was also observed that infection of older plants led to reduction in fruiting, shooting, tillering and in case of severe infection, the rhizome wrinkled and gradually dies.

Varma (1962a) observed that the yield loss caused by CdMV is totally related to the number of years the plants had been infected. The plantations which were infected by CdMV for more than 3 to 5 years became unproductive.

Capoor (1969) observed that yield loss due to Katte disease in cardamom plants ranged from 10 to 67.56, 25.71 to 91.9 and 82.23 to 97.78 per cent during the first, second and third year respectively. The yield loss due to the disease totally depends upon the stage of the crop at the time of infection and the duration of infection. It was also observed that when the infection took place at seeding stage or early pre-bearing stage, the loss was almost total (Samraj, 1970).

Deshpande *et al.* (1972) found that Katte disease of cardamom was the reason for low yield and rapid decrease in the area under cardamom plantation in Southern India. Naidu *et al.* (1981) reported that the maximum reduction in growth and yield of cardamom was in plants that were inoculated with the virus in the same year of planting. Furthermore, 15, 30 and 90 per cent reduction in yield was observed in the first, second and third year of inoculation respectively.

Naidu *et al.* (1983) reported that due to infection of Katte, there was a reduction of 25.6 per cent in tiller production, 5.6 per cent in height of clump, 18.6 per cent in leaf area and 63.3 per cent in number of capsules in infected plants when compared with healthy plants.

Venugopal and Naidu (1984) reported that bearing plants infected with katte showed 38 per cent drop-off in yield during the first year of infection under monocrop situation, while 62 per cent and 68.7 per cent in the second and third year of infection respectively. The infected plants died after 3-5 years of infection.

Siddaramaiah *et al.* (1991) examined the effect of infection on the capsule, seed development and oil content, and found that within two years of infection, there was about 50 per cent decrease in fresh weight of capsules while clumps that were infected for six months, showed 39 per cent reduction in the fresh weight of capsules. Furthermore, there was a reduction of 7.06 and 2.24 per cent in oil

content of capsules harvested from two years and six month old clumps infected with katte respectively as compared to healthy clumps.

As per the survey conducted by Biju *et al.* (2010) in the cardamom growing tracts of Karnataka and Kerala, it was reported that the disease incidence ranged from 0 to 85 per cent and incidence of disease was much higher in the cardamom plantations of Karnataka compared to Kerala.

2.2. Symptoms of Katte disease

Uppal *et al.* (1945) revealed that the first visible symptom appears on the youngest leaf of the infected plant. The infected leaf shows the chlorotic flecks, which later develops into pale-green stripes running parallel from the midrib to the margin over the entire surface of leaf tissue and shows a mosaic pattern on fully developed stage of the disease. It was also observed that the new daughter clumps from the infected mother clumps had stunted growth with gradual decrease in their productivity followed by death.

Symptoms of Katte mosaic disease on the young leaves were characterized as interrupted pale green stripes, which extend along the veins and parallel to each other from midrib to margin of the leaf. In advanced stage, the pale green stripes are distributed evenly over the leaf surface, showing a mosaic symptom and all developing leaves subsequently showed the pale green stripes. However, there was no malformation of any plant part but the infected plants showed a gradual reduction in size of leaves and pseudostems (Capoor, 1967).

Rao and Naidu (1973) observed that the infected plants were stunted in growth and mosaic symptoms appeared on the leaf sheath and pseudostem. However, infection occurring in mature plants showed fewer stunted pseudostems. The fruit bearing capacity of the infected plant gradually declined whereas

seedling stage infection resulted in failure in flowering and fruiting leading to decrease in productivity (Nambiar *et. al.,* 1975).

Rao (1977) identified and classified three isolates of katte infected plants based on the symptoms produced and labeled them as A, B and C. The isolate A had dark green longitudinal stripe whereas isolate B had broken bands of dark green colour with chlorotic stripes. Isolate C showed stunted growth with granular mosaic pattern on the leaf.

Dimitman *et al.,* (1984) reported symptoms of *Katte mosaic virus* as downward movement of new shoots including mild chlorosis with green-yellow mottling and reduced growth rate. Venugopal (1995) reported the systemic nature of infection, which gradually infect all the tillers in a plant. In advanced stage, the infected plants produce small and thin tillers with few shorter panicles and degenerated gradually.

Biju (2010) surveyed the cardamom plantations of Karnataka and Kerala and reported that the symptom of cardamom mosaic virus varies with variety and location. The survey revealed that the variety ICRI-3 and IISR Kodagu Suvasini showed similar symptoms which include prominent light green and yellow patches along the vein while Njallani green gold showed light green with prominent chlorotic streaks on leaf of infected plants of cardamom cultivated in Karnataka region. But in Kerala and Tamil Nadu region, Njallani green gold showed prominent light green or yellow mosaic patches along the veins.

Siljo *et al.* (2013) observed that among the different symptoms of *Katte mosaic virus* collected from different locations of Kerala and Karnataka regions, the samples collected from Karnataka region show mild mosaic mottling symptom with yellow mosaic pattern running parallel to veins. Whereas, Samples collected from Kerala and Tamil nadu show severe mosaic mottling on the leaves.

2. 3. Etiology of Katte disease of cardamom

The earlier investigations show that Katte disease was confined to soil. But Mollison (1900) reported that degenerative nature of the disease can be brought about by continuous cultivation of cardamom under same agronomic conditions without changing the propagation technique. Desai (1914) reported that the reason of katte disease was continuous application of inferior quality leaf manure till the harvest.

Sahasrabudhe (1917) examined the biotic and abiotic factors responsible for the development of katte disease and reported that none of the biotic and abiotic factors were responsible for the deterioration in health of cardamom plants.

Sahasrabudhe and Bapat (1929) reported that soil infected with *Calpidium striatum* (Strokes), a soil protozoan, was the reason for mosaic disease of cardamom but later, Thomas (1939) reported that Katte disease of cardamom was caused by virus transmitted through infected rhizomes which were used as planting material.

Uppal *et al.* (1945) reported that virus was the major causal organism of Katte disease of cardamom which is transmitted through banana aphid *Pentalonia nigronervosa* Coq.

2. 4. Transmission of *Cardamom mosaic virus*

2. 4. 1. Transmission through seed

Thomas (1938) reported that the disease was transmitted through rhizomes collected from the virus infected plants but not through seeds. Rao and Naidu (1973) also reported that the virus was not transmitted through seeds but the seeds collected from virus infected clump showed less germination percentage (about

38.2%) when compared to the seeds collected from the healthy clumps (about 92%).

2. 4. 2. Transmission through mechanical sap

Uppal *et al.* (1945) observed that the *Katte mosaic virus* of cardamom is not sap transmissible to host. Rao (1972) and Rao and Naidu (1973) reported that the mosaic virus of cardamom can be sap transmitted to *Phaseolus vulgaris cv. Dwarf Stringless, Vigna sinensis cv. Black eye* and *Crotalaria striata.* Later Rao (1977) reported that the virus can also be transmitted to *Cucumis sativus cv.* Green Long.

Rao and Naidu (1973) reported that the young leaves infected with *Katte mosaic virus* were freeze at 4°C for 24 hours and then ground in activated charcoal and 0.01 M phosphate buffer pH 7 at 10-12°C which was used as inoculums for transmission of the virus.

2. 4. 3. Transmission through insect vectors:

Uppal *et al.* (1945) first reported that Katte virus was transmitted by *Pentalonia nigronervosa* Coq. The insect breeds underneath loose leaf sheaths on the stem and their large colonies were found on decaying cardamom plants. The aphids also infect and colonized on banana plants.

Varma and Capoor (1958) observed that the katte disease of cardamom cannot be transmitted by mechanical means or by plant to plant touch. Furthermore, it was also observed that a single aphid *Pentalonia nigronervosa* was capable of transmitting the disease to healthy plants. Siddappaji and Reddy (1972) observed that *Pentalonia nigronervosa f. caladii* Vander Goot. was the main vector for *Katte mosaic virus.* Rao (1972) reported that several aphids like *Pentalonia nigronervosa f. caladii* Vander Goot., *Aphis nerii*, *Macrosiphum sonehi*, *Greenidia artocarpi* and *Aphis gossypii* were normally infecting cardamom.

Rao and Naidu (1974) reported that 14 aphids species can transmit *Katte mosaic virus* of cardamom. They are *Pentalonia nigronervosa f. caladi, Aphis gossypii* (Okra strain), *Aphis gussypii* (Commelina strain), *Macrosipltum pisi, Greenidia artocarpi, Schizaphis graminum, Schizaphis cyperi, Aphis nerii, Aphis craccivora, Macrosiphum rosaeformis, M. sonchi, Brachycaudus helichrysi* and *Aphis rumieis.*

2. 4. 4. Virus-Vector Relationships

Varma (1956) reported that there was no inherited transmission of the virus by *Pentalonia nigronervosa,* but the nymphs could take the virus from diseased plants whereas, Varma and Capoor (1958) and Varma (1962b) reported that the virus was transmitted in semi persistent manner.

Varma (1962b) reported that a single *Pentalonia nigronervosa* (viviparous and alate female) can transmit the virus but a larger number of aphid population ranging from 15-20, showed a higher percentage of viral infection.

Rao (1977) found that 100 per cent infection was observed when five aphids (*Pentalonia nigronervosa f. caladii*) were used at the same time for transmission of virus. It was also noticed that aphids transmitted the disease within 3-5 minutes, if they were given pre-acquisition fasting period of two hours.

Rajan (1981) reported that the nymphs of all the instars of *Pentalonia nigronervosa* were capable of transmitting katte disease and the percentage of transmission increases with the increase in age of the nymph which shows positive correlation. Dimitman (1981) reported that *Pentalonia nigronervosa* and *Toxoptera spp.* can also transmit the *Cardamom mosaic virus* with an acquisition period of 1 to 1½ hour.

2. 5. Disease development among infected plants

Varma (1956) reported that virus takes about 21-46 days for developing symptom after transmission by aphids at any stage of plant growth and development except at seedling stage. Dimitman (1981) observed that after the inoculation of cardamom mosaic virus on a healthy plant, it takes about 40-46 days for expression of symptoms on the plant.

Naidu *et al.* (1983) studied the time taken to develop visible symptoms after infection in different months under field conditions and reported that, virus requires 34 to 97 days in different months for development of symptoms whereas during the months of January to march, the virus required a prolonged incubation period of 63 to 97 days.

Naidu and Venugopal (1984) reported that the development of disease was directly related to plant growth and it requires 20-114 days of incubation period for development of symptom in host plants.

2. 6. Host Range for *Katte mosaic virus*

Varma and Capoor (1958) reported that *Katte mosaic virus* had no effect *on Amomum sp.* whereas Samraj (1970) observed the symptoms of katte disease on *Amomum sp.* including *A. cannecarpum* and *A. involucartum.*

Rao and Naidu (1973) reported that *Amomum sp., Amomum cannecarpum , A. involucartum* and *Zea mays* are the collateral hosts for *Katte mosaic virus.* The other collateral hosts for *Katte mosaic virus* are *Amomum subulatum* and *Alpinia sp.* (Rao, 1972); *Alpinia neuten* (Viswanath *et al.,* 1973; Viswanath and Siddaramaiah, 1974) and Arrow root (*Maranta aurundiacea*) (Siddaramaiah *et al.,* 1986)

2. 7. Epidemiology and Disease Spread:

Deshpande *et al.* (1972) reported that the rate of spread of katte disease is very low and spread of disease is totally internal within plantation and there is negative correlation between the aphid population and extent of disease spread.

Rao (1977) reported that use of katte infected sucker and rhizomes as planting material are the primary source of disease spread whereas secondary spread of disease is through insect vector *Pentalonia nigronervosa.*

2. 8. Purification of *Katte mosaic virus* from infected plants

Naidu *et al.* (1981) and Naidu and Venugopal (1984) attempted to partially purify *cardamom mosaic virus* (CdMV) from the infected leaf of cardamom. Gonsalves *et al.* (1986) purified the CdMV from the infected leaf of cardamom collected from a single plantation in Guatemala. The young to almost mature leaf showing prominent mosaic symptom were collected and grounded in liquid nitrogen. The virus was extracted in 0.5 M KP (Potassium Phosphate) buffer, pH-7.1 containing 0.01M EDTA and 0.1 per cent β-mercaptoethanol and Triton X-100 was used as clarifying agent. The extract was ultra centrifuged for purification of the virus. Finally the virus was concentrated on 10% sucrose cushion in 0.025 M KP buffer, pH 7.1 containing 1M urea and then virus was purified by Cesium Sulphate (Cs_2SO_4) gradient centrifugation.

Jacob and Usha (2001) purified the CdMV from infected cardamom leaf by using 0.1 M sodium borate buffer (pH-8.2) containing 2% PVP, 0.1% EDTA, 0.1% *β*-mercaptoethanol and 0.5M urea. The extract was clarified using 9:1 mixture of chloroform and n-butanol and then centrifuged over 20 per cent sucrose cushion. The virus was further purified and concentrated by sucrose gradient centrifugation.

2. 9. Characterization of *Katte mosaic virus*

Naidu *et al* (1981) collected Katte infected cardamom plants and characterized into ten isolates based on symptoms produced on the young and matured plant, plant morphological characters and transmission efficiency.

Gonsalves *et al.* (1986) reported that the three molecular weights of *Cardamom mosaic virus* coat protein were, 37.5, 32.2 and 29 kDa in 12 per cent Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Jacob and Usha (2001) isolated the virus coat protein and ran on 12 percent SDS-PAGE and reported that the molecular weight of coat protein was 38 KD.

Jacob and Usha (2002) expressed the coat protein (CP) gene of *cardamom mosaic virus* in *E. coli* and the recombinant protein which was used as antigen for production of polyclonal antibody against *Cardamom mosaic virus* in rabbit was purified by affinity chromatography. The viral nature of the expressed coat protein was confirmed by western blot. Positive reaction was observed with the IgG antibody produced against cardamom mosaic virus. Filamentous aggregates were formed in *E. coli* colonies due to expression of coat protein gene observed under immuno-gold electron microscopy and were of 100-150 nm in length.

2.10. Electron Microscopic studies of *Katte mosaic virus*

Naidu *et al.* (1981) and Naidu *et al.* (1983) observed the particle morphology of *Katte mosaic virus* by electron microscopy and reported that virus had slender flexuous rod shaped particles measuring 650 nm in length and 10 to 12 nm in diameter. Based on particle morphology, the virus was classified under *Potato virus Y* group. Dimitman *et al.* (1984) observed ultra thin sections of leaf taken from the infected plant showing prominent symptoms of mosaic and reported that the flexuous

rods, helical, symmetrical structure of virus and the cells of infected plants contained pin-wheel shaped inclusion bodies.

Gonsalves *et al.* (1986) observed the extract of young unrolled infected leaf under an electron microscope and reported the presence of flexuous rod shaped particles and pinwheel shaped inclusion body from the cells of infected plant which is typically formed by the potyviruses.

Jacob and Usha (2002) observed the purified virus under the electron microscope and reported that the virus has flexuous filamentous particles of about 800 nm in length which is typically shown by the members of *Potyviridae.*

2.11. Detection of *Cardamom mosaic virus*

Rao and Naidu (1973) reported that katte disease samples can be stained by acidified phloroglucinol or Giemsa stain. The transverse section of the midrib of healthy and infected leaf tissue when stained with phloroglucinol or with giemsa stain showed that the phloem and xylem of the healthy tissue were deep purple stained while the phloem of infected leaf tissue was stained light blue whereas xylem vessels did not show any colour change in any of two stains.

Rao and Naidu (1973) also stained the inclusion body of the Katte infected young leaf by trypan blue and found that the size of inclusion body in infected plant varies from 5-50 nm in diameter which may be oval or round and 1-15 nm long and may have plate or rod like inclusion body.

Gonsalves *et al.* (1986) raised the antiserum against the CdMV and used in direct and indirect double sandwich ELISA for detection of virus. In direct ELISA 1:800 diluted alkaline phosphatase conjugated cardamom mosaic virus IgG gave the positive result whereas in indirect ELISA 1:1000 diluted alkaline phosphatase conjugated antirabbit IgG showed the positive result.

Biju *et al.* (2010) extracted the total RNA from the infected leaf of cardamom using TRI-reagent (Sigma-Aldrich) and detected the CdMV through single step reverse transcription polymerase chain reaction (RT-PCR) by using primers designed for targeting the conserved region of the coat protein of virus and the reaction was called as positive because of the presence of 800 bp amplicon in infected leaf samples with no such amplicon observed in healthy samples.

Jebasingh *et al.* (2011) detected the CdMV in the plant genome. The integrated DNA of the virus was found identical with the 3'-nuclear inclusion body (NIB) which was RNA dependent RNA polymerase gene of CdMV infecting cardamom.

Siljo *et al.* (2014) developed the SYBR based single step real time PCR for the detection of CdMV by using a primer, targeting the coat protein of the virus. This was the first report of real time PCR for detection of CdMV in cardamom.

Tiwari *et al.* (2016) used serological tests such as ELISA and DIBA for the detection of CdMV from the infected samples collected cardamom plantations of Karnataka. The polyclonal antibody were produced against the virus and used in DAC-ELISA and DIBA. The crude extract of infected leaf samples having 1µg antigen were showed positive reaction with 1: 500, 1:1000 and 1:2000 dilution of primary antibody raised against virus. Whereas, 1:500 and 1:1000 dilution of primary antibody with 1:1000 dilution of secondary antibody showed the positive result in DIBA and reported that ELISA is the best method for the detection of CdMV. The method was also used for the detection of CdMV in the tissue cultured plants.

2.12 Serological relationship of *Katte mosaic virus* with other Potyviruses:

Koenig (1981) found that indirect and direct ELISA can be used to examine the serological relationship of plant viruses belonging to potyvirus group.

Dimitman *et al.* (1984) used indirect ELISA with antiserum of *Potato virus Y* (PYV) to determine the serological relationship of CdMV with PYV. The crude protein extracted from the infected leaves of CdMV showed positive reaction with the antiserum of PYV in indirect ELISA. The result of indirect ELISA revealed that CdMV had some relationship with PYV.

Gonsalves *et al.* (1986) performed the indirect ELISA to determine the serological relationship of cardamom mosaic virus antiserum with 16 isolates of potyvirus and reported that *Zucchini yellow mosaic virus* (ZYMV), *Papaya ring spot* virus type w and p, NL-8 isolates of *bean mosaic virus* gave positive reaction in indirect ELISA whereas *Cowpea aphid borne mosaic virus* (CAMV), *Clover yellow vein virus* (CYVV) and *Black eye cowpea mosaic virus* (BICMV) showed positive reaction.

Smitha (2004) used indirect ELISA to determine the serological relationship of CdMV with members of other viruses. The antisera of different viruses were used in indirect ELISA to cross react with the crude extract of katte infected cardamom leaves. Based on the absorbance reading, the reaction was called as positive when the absorbance value of infected samples was more than twice the absorbance value of healthy samples. The result of indirect ELISA revealed that CdMV was serologically related with *Banana bract mosaic virus* which is a putative member of potyvirus group.

2.13. Molecular diagnostics of plant viruses:

The early and specific detection of plant diseases is a most important step in any crop protection system. Plant diseases can be effectively managed by introducing the control measures at an early phase of disease development. There are some biological techniques used for disease diagnosis and pathogen detection, but the process is slow and cannot be used for large-scale application (Miller and Martin,

1988). However, some of the viral diseases can be diagnosed based on their visual symptoms. But relying on the visual symptoms for managing the viral diseases is not advisable because they may be symptomless or there are number of different viruses which produce similar symptoms on the plant. Molecular test is required for their diagnosis (Webster *et al.,* 2004).

Recent advancement in biotechnology and molecular biology played a vital role in the development of several rapid, specific and sensitive molecular techniques for easy and early detection of plant viruses. These molecular diagnostic techniques are divided into two groups. First in serological techniques which includes ELISA which has been used from many years for the detection of viruses and second is nucleic acid based techniques which include Polymerase chain reaction (PCR) like reverse transcriptase PCR (RT-PCR) *etc*., which are extremely useful for detection of new viruses or viral strains (Makkouk and Kumari, 2006).

2.13.1. Molecular diagnostics of plant viruses using ELISA

The most widely used serological technique for the detection of plant viruses is ELISA which is based on the antiserum produced against a particular virus protein. This technique was first introduced to plant pathology by Voller *et al.* (1976) and Clark and Adams (1977), where virus specific antibodies were linked to the enzyme alkaline phosphatase and termed as an antibody sandwich assay (Gould and Symons, 1983). The method was extremely sensitive for detecting viruses from the infected tissue of leaves, bulbs, and tubers. ELISA is a sensitive method which can be used to detect viruses from the tissues such as tuber, leaves and bulbs *etc.* (Cooper and Edwards, 1986).

Kim *et al.,* (1987) reported that yellowing and vein bending on the leaf surface of pepper plants was caused by virus. The leaf extract was taken from the infected plant and the extract reacted strongly with antiserum of potato virus Y in

ELISA which confirms the viral nature of disease. However, it didn't show any reaction with the antiserum of *Cucumber mosaic virus, tomato spotted wilt virus, tobacco etch virus* and *tobacco ring spot virus* in ELISA

Fernandez *et al.* (1988) used heterologous sandwich ELISA with antibody obtained from hyper immune ascetic mice for effective detection of *tobacco mosaic tobamo virus* in tobacco.

Arunasalam and Pearson (1989) used ooze coming out of the cut surface of cymbidium leaf as antigen in ELISA for detection of *Odontoglossum ring spot tobamo virus* and reported that the method was quick but required more number of infected leaves to increase the concentration of virus in the ooze for better result in ELISA.

Brown and Poulos (1989) reported indirect ELISA with antibody produced against viral coat protein of *Lettuce infectious yellows virus.* The samples were collected from the green house and field inoculated plants were used in indirect ELISA for the detection of LIYV. The method was also used for the detection of LIYV from symptomatic and asymptomatic weed and cultivated plant species collected from infected fields of Yuma and Central Arizona. The LIYV were also detected from the infected plants of muskmelon plants using indirect ELISA.

Rowhani *et al.* (1992) reported that ELISA can readily detect the *Grapevine fan leaf virus* (GFLV) and *Tomato ring spot virus* (TomRSV) in infected grapevines as well as determine the influence of season, host genotype, virus isolates and sample tissue on ELISA detection. There were no difference in GFLP ELISA results when compared with different cultivars and viral isolates but it showed seasonal difference in GFLP ELISA result whereas there was no difference in the ELISA result of TomRSV over season and sample tissue, it produced relatively similar results in ELISA.

Huguenot *et al.* (1996) optimized the condition of DAC-ELISA for diagnosis of *tomato spotted wilt virus* (TSWV) with monoclonal antibody (mAb) 6C12 raised against Nigerian isolates and reported that virus had serological relationship with potato yellow mosaic virus. The method was also compared with Dot-hybridization technique and reported that ELISA with 6C12 monoclonal antibody is more sensitive for the detection of TSWV virus.

Ismail (1997 and 1998) artificially infected leaves and tuber of potato cultivar with *Potato virus Y*. The crude protein was extracted from the artificially inoculated leaves and tuber which was then used in ELISA for the detection and quantification of virus. He reported that ELISA can detect the virus from 3 day inoculated seedling of potato which was 5-7 days earlier than the development of symptom. The virus can also be detected from the infected tubers after 55 days of planting which shows that ELISA is the rapid and most sensitive method for the detection of PVY from infected plants of potato.

Skomra (1997) reported the effect of sampling time and use of different parts of hob plant on ELISA for the detection of *hop mosaic carla virus* (HMV) and *Prunus necrotic ringspot ilar virus* (PNRSV). The HMV showed optimal ELISA reading with crude extract of older leaf infected with virus. Whereas, tip of stem, young leaf and flower are the best materials for the detection of PNRSV through ELISA. It was also observed that the samples collected for detection of both the virus at the end of growing season showed poor result in ELISA.

Yardimci and Bostan (1999) used ELISA for investigating the disease incidence of viruses such as *Potato virus X* and *Y* (PVX and PVY) and *Potato leaf roll virus* (PLRV) infecting potato plants cultivated in Erzurum region of Turkey. A total of 270 samples were collected and tested in ELISA and found that potato plants in Turkey were mostly infected by PLRV with disease incidence of 42.2 percent followed by PVX, 38.3 per cent and PVY, 7 per cent.

Bhat *et al.* (1999) compared three serological techniques ELISA, DIBA, EBIA (Electro Blot Immuno Assay) for detection of potyviruses and reported that through ELISA, upto 1 ng of virus can be detected which was much lower as compared to DIBA and EBIA, using which up to 100ng of virus can be detected.

Ling *et al.* (2000) developed the polyclonal antibody against Clusterovirus-3 linked with grapevine leaf roll disease of grape by expressing the coat protein gene in the *E. coli* identified after screening the cDNA library. DAC ELISA was performed to determine the specificity of the antiserum to detect the virus. The result showed that the virus can be detected easily with the As163 (coating agent)-Mab system in DAC-ELISA.

Jain *et al.* (2000) standardized the DAC-ELISA protocol for the detection of *Peanut stripe potyvirus*. DAC-ELISA was also used for determining the serological relationship of *Peanut stripe potyvirus* with other member of potyvirus and reported that the *Peanut stripe potyvirus* had serological relationship with *Cowpea aphid-borne mosaic (CABMY)* and *Soybean mosaic* (SbMV) *potyviruses.*

Doves (2002) determined the sampling conditions for diagnosis of *Onion yellow dwarf* virus (OYDV) and *Leek yellow stripe virus* infecting garlic using ELISA technique. Through DAC-ELISA, the relative concentration of both the viruses were determined in different parts of garlic plant collected during the growing period and found that both the viruses were unevenly distributed in different parts of garlic plant. The highest concentration of the viruses was found in tip of newly developed leaves but the concentration was found to be low in older leaf samples.

Hourani and Abou-Jawdah (2003) amplified the coat protein gene of *Cucurbit yellow stunting disorder virus* using RT-PCR and the coat protein gene was expressed in the bacterial expression vector pQE-31. The recombinant coat protein was used as antigen to produce the antibody against the virus in rabbit. The resulted antibody was

used in indirect and direct ELISA with 1:1000 dilution of antibody to detect *Cucurbit yellow stunting disorder virus*. Similar method was used by Nickel *et al.* (2004) where they raised polyclonal antibody in rabbit against *Apple stem grooving virus* (ASGV) using the recombinant coat protein expressed in *E.coli* which were used as antigen. The antibody was used in plate trapped ELISA for the detection of virus with 1:1000 and 1:2000 dilution of antibody and reported that the method can be used as an additional tool for virus indexing for ASGV infected plant of apple which will help in virus free cultivation of apple.

Jain *et al.* (2005) used the gene expression system under *invitro* condition for the production of polyclonal antibody against recombinant coat protein *of Ground nut bud necrosis virus*. The antibody was used in indirect ELISA with 1:1000 dilutions for detection of *Ground nut bud necrosis virus* and *Watermelon bud necrosis virus* infecting the crops belonging to family Cucurbitaceae, Leguminaceae and Solanaceae.

Raikhy *et al.* (2007) developed the ELISA based kit for the detection of *Carnation etched ring virus* infecting carnation. The coat protein gene was cloned and expressed under *In vitro* condition in *E.coli.* The coat protein was isolated from the culture and used as antigen to develop antiserum against *Carnation etched ring virus.* The ELISA kit was also compared with the commercially available kit for detecting the *Carnation etched ring virus* and found that the antibody had good specificity and detectability and detected virus from the samples collected from the field and green house.

Eoi *et al.* (2010) developed the polyclonal antibody in rabbit to detect CMV (*Cucumber mosaic virus*) infecting yam isolates in Ghana. The coat protein was isolated from the infected leaf of yam which was used as antigen for injecting rabbit for the development of antibody. The antibody titre was determined by using PAS-ELISA and Indirect ELISA. Indirect ELISA was used for detecting the CMV infected

plants in Ghana using polyclonal antibody raised against CMV with 1:64000 dilution of antiserum.

Ray *et al.* (2015) developed the ELISA kit for easy and early detection of rhizome rot in turmeric. The polyclonal antibody was raised against *Pythium aphanidermatum* and used in standardized ELISA condition with incubation period of 16-18 hours for early detection of the pathogen.

Jooste *et al.* (2016) used BBTV specific ELISA kit for early detection of *Banana bunchy top virus* in banana. This was the first report of BBTV infecting banana plants in South Africa.

Lacroix *et al.* (2016) developed the methodology for detection of viruses using ELISA for woody perennial plant species and reported that stem extract of woody perennials can be effectively used for detection of viruses in ELISA rather than using leaf extract, thus, highlighting the importance of sampling for detection of viruses through ELISA.

2.15.2. Molecular diagnostics of plant viruses using RT-PCR

Polymerase Chain Reaction (PCR) is a simple, sensitive, adaptable and ingenious technique which is widely used for the detection of plant viruses. The viruses whose genetic material is RNA can easily be detected by modified form of PCR called as RT-PCR (Reverse Transcription Polymerase Chain Reaction). In RT-PCR, the RNA isolated from the plant is converted into cDNA (Complementary-DNA) by using the enzyme reverse transcriptase and further amplified with primers targeting the viral genome. Now RT-PCR has become the most versatile technique for the detection of RNA viruses infecting the plants (Henson and French, 1993)

Pappu *et al.* (1993) used RT-PCR to detect *Dasheen mosaic virus* (DMV) affecting the plant belonging to family Araceae. The total nucleic acid was isolated

from the infected plants and single step RT-PCR and PCR amplification were carried out using degenerate primers. The degenerate primers were designed to amplify the 3' untranslated region (UTR), conserved regions in coat protein such as WCIEN and QMKAAA boxes present in potyviruses group and 3' Poly-A tail of the genome. Whereas, the reverse primer had 21 Thymine residues with Adenine (A), Cytosine (C), or Guanine (G) at 3' end to maintain the specificity of the priming at the 3' end of cDNA containing 3' UTR and poly A-tail. The specificity of the degenerated primer were also checked with known poty-viruses like *Watermelon mosaic 2* and *Soybean mosaic virus* using single step RT-PCR. The amplified product of DMV was cloned and sequence similarity with other potyviruses showed that DMV is a diverse virus with not much similarity with any other poty-viruses.

Colinet *et al.* (1994) used combination of reverse transcriptase and PCR with degenerate primers designed from the conserved regions in the coat protein to amplify the 5' variable region of the coat protein of potyvirus infected sweet potato plant population. The cDNA amplification of two clone of sweet potato from China with degenerated primers gave 1.35 Kb bands which was associated with *Sweet potato feathery mottle virus* (SPFMV) after sequence comparison. Two bands of size 1.30 and 1.40 kb were observed which suggested that the clone were infected with more than one potyviruses.

Stevens *et al.* (1997) detected the *Beet yellows clusterovirus* (BYV) infecting the beet root and insect vector aphids (*Myzus persicae*) by RT-PCR using the primer amplifying the conserved region of the coat protein open reading frame 7 and 8. Three protocols for the isolation of total RNA were tried to extract sufficient amount of total RNA for RT-PCR, Although RNA isolation using Kit provided by Flowgen was found best for isolating RNA from the single aphid. Sixty aphid populations were fed on the infected beet root plants and tested in RT-PCR. The result of RT-PCR revealed that out of 60 aphids, 55 individuals were found positive for BYV. The 36

aphid populations were also diagnosed in TAS-ELISA with monoclonal antibody of BYV and 53 per cent of aphids showed positive result but no positive result was obtained when p-nitrophenyl phosphate was used as substrate in TAS-ELISA.

Kim *et al.* (1999) used RT-PCR assay to detect the strains of *Soyabean mosaic virus* (SMV) using the primers amplifying the cylindrical inclusion (CI) coding region present in between 4,176 to 5,560 nucleotide in the viral genome. The total RNA was isolated using Kit provided by promega. The amplification of cDNA yielded 1385 base pair DNA fragments. Furthermore it was also reported that RT-PCR was 1000 times more sensitive than ELISA and can detect the virus in 10^{-6} dilutions. The virus was classified into six groups based on the fragments obtained after restriction digestion of RT-PCR product with ECoR1.

Hung *et al.* (2000) reported a single step RT-PCR assay that can detect different strains of *Citrus tristeza virus* (CTV) infecting citrus plant using CTV specific primers. The comparison of two steps RT-PCR and ELISA with single step RT-PCR showed that single step RT-PCR was more sensitive for detecting CTV even though the concentration of virus is very low. The 3 strains of CTV detected using RT-PCR proves the accuracy of the method with CTV specific primer.

Gillaspie *et al.* (2001) reported about the RT-PCR technique for easy detection of *Cowpea aphid-born mosaic virus* which was a seed born pathogen infecting peanut and cowpea. The RNA was isolated from the seed and leaf of the infected peanut plant and RT-PCR was carried out with the primers designed to amplify the coat protein region of the virus. Furthermore, it was also observed that RT-PCR can detect the virus from one infected leaf among 99 healthy leaf samples whereas in ELISA one infected leaf can be detected among 9 healthy leaf samples which shows that RT-PCR is highly sensitive for detecting the *Cowpea aphid-born mosaic virus.* Kuroda *et al.* (2002) used multiplex RT-PCR for simultaneous detection of broad bean with virus 2, *Cucumber mosaic virus* and *Clover yellow vein*

virus using coat protein specific primer in *Gentlala* sp. The sensitivity of multiplex RT-PCR were also compared with DIBA (Dot Immuno Binding Assay) and found that RT-PCR was 10000 times more sensitive than DIBA.

Meunier *et al.* (2003) developed the multiplex RT-PCR technique for simultaneous detection of *Beet necrotic yellow vein virus*, *Beet soil borne virus*, and *Beet Virus Q* with their vector *Polymyxa betae*. The result showed that the detection capacity of RT-PCR was 128 times higher than ELISA.

Ioannis et al. (2004) developed the sensitive RT-PCR technique for the detection of strawberry pallidosis disease. For the detection of virus, primers were designed using primer3 software to amplify the coat protein region of the virus. The total RNA was isolated from the infected leaf and converted into cDNA using the kit provided by invitrogen. cDNA was used as template for the amplification of viral coat protein region by using SP-F and SP-R primer which gave 517 bp fragment related to HSP70 gene and CP5' and CPn731R primer gave 752 bp fragment which contained most of the coat protein region of the virus. The 752 bp fragments were cloned and the sequence similarity showed 99 percent similarity with *Cucumber yellows virus* and *Cucurbit yellow stunt disorder virus* belonging to genus *Crinivirus.*

Smitha (2004) used two steps RT-PCR for detection of *Banana bract mosaic virus* (BBrMV) infecting cardamom plant using the primers amplifying the conserved region of Nib gene and PCR product of 1.8 kb was obtained from the infected plant whereas no amplification was observed from the healthy plants of cardamom. The specificity of the virus were also checked by PCR amplification with primers BBrMVF and BBrMVR which resulted in the 900bp fragment which was the expected band size for BBrMV.

Yueh-Chwen *et al.* (2005) developed the degenerate primers from the NIB and coat protein region for the diagnosis of potyvirus using RT-PCR. The primer was

able to amplify the 1-1.2 kb cDNA fragment of 3' terminal region of six poty viruses. The result showed that RT-PCR with degenerate primers had satisfactory specificity and do not react with non-potyviruses which can be rapidly used for the detection of potyviruses.

Bhat and Siju (2007) detected *Cucumber mosaic virus* (CMV) and *Pepper yellow mottle virus* (PYMoV) in black pepper linked with stunt disease using the multiplex RT-PCR assay for simultaneous detection of viruses in a single tube. The primer pairs were designed to amplify 650bp from coat protein region of CMV and 450bp from the ORF (Open Reading Frame) 1 of PYMoV. The method is highly sensitive and required very small amount of samples for detection.

Lakshmi (2010) used RT-PCR for the isolation of partial coat protein gene of *Banana bract mosaic virus*. The total nucleic acid isolated and cDNA was synthesized using RT-PCR kit. The PCR amplification was carried out with specific primer Bract1 and Bract2 and a product of 604 bp was obtained from the infected plant samples. The fragment was cloned and the sequence analysis showed similarity with BBrMV coat protein and potyvirus coat protein gene.

James and Launa (2011) developed the RT-PCR protocol for the detection of 11 viruses infecting potato including *Potato tuber spindle tuber viroid*. Gene specific primers amplifying the coat protein of the viruses were used in RT-PCR for the detection of viruses in infected samples of potato and reported that the assay is versatile, convenient and cost effective for detection of viruses infecting potato which can be useful for potato seed certification agencies.

Noveriza *et al.* (2012) used RT-PCR for the detection of potyvirus associated with the mosaic disease of patchouli using degenerate primers targeting the coat protein gene of the potyvirus. Product of 800 bp was obtained after PCR amplification which was sequenced and similarity search was done using BLAST.

The result showed that the virus was closely related with *Telosma mosaic virus* (TeMV) and *Passion fruit woodiness virus* (PaWP) which also belongs to potyvirus group.

2.16. Sequence analysis for determination of variability

Comparison of amino acids and nucleotide sequence with published sequences is a preeminent method for analyzing the variability and genetic relationship among the viruses. The coat protein sequences had been used for differentiating the viruses and their strains which belongs to potyvirus group. The coat protein sequences of different members of viruses show only 38 to 71 per cent similarity but remarkably differ in length and amino acid sequence. Furthermore, the coat protein sequence of different strains of a virus shows more than 90 per cent similarity with similar amino acid sequence (Shukla and Ward, 1988). A large number of sequences related to potyviruses are available in the database which are useful for distinguishing closely related virus, their strain or different isolates by comparing the amino acid or nucleotide sequence of coat protein and 3' untranslated region (UTR).

Batson *et al.* (1994) studied the variability of *Papaya ring spot virus* found in Australia. The coat protein gene of virus was isolated from six isolates of Australia and three Asian isolates which were sequenced and compared with the previously reported sequence of *Papaya ring spot virus*. It was observed that there was 12 per cent variability in nucleotide sequence of Asian isolates as well as Asian and Australian isolates. The variability was also observed in the amino acid sequence of Asian isolates but no significant variation was observed in the amino acid sequence of Australian isolates.

Thomoson *et al.* (1995) performed RT-PCR for the detection of *Zucchini yellow mosaic virus* (ZYMV) in cucurbits by using the primer amplifying the Nib

region of viral genome. A 300 bp fragment was obtained after PCR amplification which was sequenced and Nucleotide sequence analysis using BLAST were performed. The result showed 93.7 to 100 per cent similarity with ZYMV. The multiple sequence alignment of nucleotide showed 74-10 per cent similarity with coat protein of ZYMV.

Jain *et al.* (1998) cloned and sequenced the 1.7 kb fragment of *Papaya ring spot virus* which contains the complete coat protein region, part of Nib gene, and 3' UTR region for characterization of two strain of virus type P and W. The nucleotide sequence analysis showed that 96 percent similarity in 3' UTR region of type P and W with 96 per cent similarity in nucleotide sequence of coat protein and 87 per cent similarity in amino acid sequence.

Berger *et al.* (1999) characterized the different strains of *Bean common mosaic virus* (BCMV) and one strain of *Bean common mosaic necrotic virus* (BCMNV) using Phylogenetic analysis of 3' terminal sequence containing coat protein and 3'UTR region. The result of nucleotide sequence analysis revealed that BCMNV is different from BCMV and also found that the *Dendrobium mosaic virus, Black eye cowpea mosaic virus* and *Peanut strip virus* are different strains of BCMV.

Lin *et al.* (2000) isolated the complete nucleotide sequence of coat protein gene from five isolates of *Zucchini yellow mosaic virus* (ZYMV) from Taiwan using RT-PCR and sequenced. The phylogenetic analysis of the actual sequence showed that four genotypes of ZYMV were found in different geographical areas in which Taiwan isolates of ZYMV were classified into genotype-1. The nucleotide comparison of coat protein gene of five isolates of ZYMV collected from Taiwan showed 92.8 to 98.7 per cent similarity whereas 96.4 to 99.3 per cent similarity in amino acid sequence. Jacob and Usha (2001) cloned and sequenced the 1.8 kb fragment of cardamom mosaic virus having coat protein region, Nib gene and 3'-UTR region using RT-PCR. The sequence analysis of the fragment showed that virus

has single continuous ORF with 529 amino acid sequence excluding the UTR region having 207 nucleotide sequences. The GDD motifs were found in the Nib region of virus which is associated with the viral polymerase and two highly conserved amino acids found in the potyviruses which were involved in the virus assembly. The phylogenetic analysis and multiple sequence alignment of the 1.8 kb fragment showed that the virus belongs to potyvirus group and new member of genus *Macluravirus.*

Nie and Singh (2002) isolated the sequence of 5'UTR region and P1 of *Tobacco veinal necrosis strain* and *Tuber necrosis strain* of *Potato virus Y* group of Europe and North America. The sequence and phylogenetic analysis of 5'UTR and P1 region of virus showed 98 per cent similarity with isolates of Europe and North America. The multiple sequence alignment of sequence revealed that the Tuber narcosis strains were probably evolved from the *Tobacco veinal necrosis strain.*

Jacob *et al.* (2003) used RT-PCR for the isolation of coat protein and 3' UTR region of cardamom mosaic virus which was cloned and sequenced from seven isolates. The sequence comparison and phylogenetic analysis showed that *Cardamom mosaic virus* is a highly variable member of potyvirus group due to variation in the coat protein and 3'UTR region among the isolates collected from different locations in South India.

Smitha (2004) reported the sequence data for *Banana bract mosaic-cardamom mosaic virus* (BBrMV-CdM) from cardamom plant. The sequence analysis of cloned DNA fragment of about 1783 base pairs revealed that the virus have single ORF of 1543 with one stop codon TAA (ochre). The multiple sequence of the cloned DNA fragment showed that Nib gene was the highly conserved region in the several potyviruses whereas major functional protein which is highly conserved among the potyviruses was found in the BBrMV-CdM DNA fragment.

Siljo *et al.* (2013) isolated the coat protein gene of *cardamom mosaic virus* from six symptomatologically different isolates of cardamom collected from different cardamom growing regions of India. The coat protein gene was cloned and the sequence comparison results revealed 74.8 to 99.3 per cent similarity in nucleotide sequence whereas 80.9 to 99.2 per cent similarity in amino acid sequence of six isolates. Based on the similarity the isolates were characterized into 3 groups in which isolates collected from Karnataka except Sirsi were categorized into group one with 90.2 to 99 per cent similarity, whereas isolates from Kerala and Tamil nadu were categorized into group three with 87.1 to 98.9 per cent similarity. Sirsi isolates of Karnataka were categorized into group two. The multiple sequence alignment and phylogenetic analysis with amino acid sequence of coat protein revealed that the isolates belonging to group one and group three are clustered together whereas the group two isolates show distinct relationship with group one and three.

Materials and Methods

3. MATERIALS AND METHODS

The study on 'Molecular characterization of *Katte mosaic virus* of cardamom (*Elettaria cardamom* Maton)' was carried out at the Centre for plant Biotechnology and Molecular Biology (CPBMB), IT-BT complex, College of Horticulture, Kerala Agricultural University (KAU), Vellanikkara during the period from August, 2014 to July 2016. The objective of the study was to develop serological and PCR based methods for identification of *Katte mosaic virus* of Cardamom (*Elettaria cardamomum* Maton.). Materials used and methodologies adopted for study was described in this chapter.

3.1. Materials

3. 1.1. Collection of Diseased and Healthy Plant Samples

Cardamom (*Elettaria cardamom* Maton) 'Njallani green gold' is a popular variety of cardamom in Kerala. The katte infected and healthy plants of this variety was collected from the different location of Wayanad district of north Kerala and Idukki district of south Kerala. The diseased samples were collected based on their symptom such as mosaic pattern with chlorotic streak on the leaves and on the pseudostem. The details of the collected samples used for this study are presented in Table 1.

3.1.2. Chemicals and Glassware

The chemicals used in the study were of Molecular grade obtained from various companies like Sigma-Aldrich, Genei, Merck, Sisco Research Laboratories Pvt. Ltd. (SRL), Thermo-scientific, Ambion-USA, Invitrogen and Himedia. Chemicals like Tris- free base, EDTA, Sodium chloride (NaCl) extra pure, Chloroform, Silver nitrate, Ammonium persulphate (APS), ELISA-kit, Diethyl pyrocarbonate (DEPC) and Sodium dodecyl sulfate (SDS) was supplied by Himedia, Mumbai whereas, Elusion kit and Trizol were supplied by Sigma-Aldrich. Potassium

Table 1: Details of diseased and healthy cardamom plants collected from different locations of Kerala

Sl. No.	Place	District	Symptom	No. of sample collected	
				Infected	Healthy
1.	Cardamom Research Station, Pampadumpara	Idukki	Mild mosaic mottling on young leaf and on pseudostem	2	1
2.	Indian Cardamom Research Institute, Myladumpara	Idukki	Mild mosaic mottling seen on the plants	2	1
3.	Farmer's field, Paravalam	Idukki	Mild mosaic mottling	1	1
4.	Farmer's field, Irettyar	Idukki	Mild mosaic mottling	1	1
5.	Farmer's field, Ambalavayal	Wayanad	Severe mosaic patterning on the leaves	1	1
6.	Elambillari estate, Meppadi	Wayanad	Severe mosaic patterning on the leaves	1	-

phosphate monobasic (KH_2PO_4; 99 %), Potassium chloride (KCl- 99 %), Glycine, Formaldehyde (40 %) was purchased from Merck India Pvt. Ltd. RNase ZAP^{TM} and Nuclease free water was supplied from Ambion, Inc, USA and Tetra methyl ethylene diamine (TEMED-ultrapure) was supplied from invitrogen. Polyethylene glycol (PEG) molecular weight-6000, ODD-kit, Twin-20, Protein loading dye (6X), BSA and Agarose was supplied from Bangalore Geni ^{TM} Pvt. Ltd whereas Isopropanol (100 %), (3-[N-Morpholino] Propane Sulfonic Acid (MOPS 99.5%), β-Mercaptoethanol, Acrylamide, Bis-acrylamide, Sulfuric acid (H_2SO_4;99 %), Sodium hydroxide pellets, Diethyl amine, p-Nitro phenyl Phosphate (PNPP), Protein ladder (14-66 KDa), DNA ladder (100 bp plus and 1 kb plus) ethanol, urea and gel loading dye (6X) was purchased from Sisco Research Laboratories Pvt. Ltd. (SRL). The C-DNA kit required for first strand synthesis from total RNA was purchased from Thermo-scientific.

The glass wares used in the present study were purchased from Borosilicate Pvt. Ltd. and Plastic products such as Petri plates, micro-titer plates (96 wells) for ELISA was obtained from Tarson Products Pvt. Ltd. All the experiments were carried out at room temperature and double autoclaved distilled water was used for the preparation of reagents.

3.1. 3. Laboratory equipments

The equipments available at Centre for Plant biotechnology and Molecular Biology were utilized for the work while the bioinformatics software's were accessed from Distributed Information Centre (DIC) of college of Horticulture. Equipment such as High speed refrigerated centrifuge, UV-transilluminator, gel rocker, Dry bath, Thermo cycler, Nanodrop, Gel doc XR, Hot air oven, Laminar air flow, Microwave oven, Autoclave, SDS-PAGE unit, Electrophoresis unit, ELISA reader *etc.* are available at CPBMB and are utilized for the study. The details of the laboratory equipments used in the study are provided in **Annexure-I.**

3.2. Methods

3.2.1. Maintenance of Diseased and healthy plants

Diseased and healthy plants of cardamom were maintained under insect proof net. The diseased plants were planted 100 m apart from the healthy plants. The diseased and healthy plants of Idukki were collected and planted in the month Feb., 2015 whereas diseased and healthy plants of Wayanad were collected and planted in the month of Sep., 2015. The plants were maintained as per the Package of practices of Kerala agricultural university and irrigated twice in day.

3.2.2. Detection of *Cardamom mosaic virus* by ELISA

3.2.2.1. Purification of viral particle from infected plants

Isolation of viral protein is a pre-requisite for the antibody production. Viral protein from the infected leaves was extracted by using method developed by Gonsalves *et al.* (1986) with slight modification.

Reagents

1. Extraction buffer
2. Re-suspension buffer
3. Chloroform: N- Butanol mixture (1:1)
4. 0.2 M Sodium Chloride (NaCl) extra pure
5. Polyethylene Glycol (PEG)-(Mw-6000)
6. PVP

Chemical composition of buffers are given in **Annexure-II**

Procedure

1. 100 g of fresh leaf samples were collected and stored in at -80 ^{0}C for 1 hour.

2. Midrib from the leaves had been removed and pulverized with liquid nitrogen in a prechilled mortar and pestle.

3. The pulverized tissues were then mixed with 1.5 volumes (1g/1.5ml) of extraction buffer. The Homogenate was squeezed through double layer muslin cloth and the debris was re-extracted with one volume of same buffer and transfered into 2 ml centrifuge tubes.

4. The filtrate was then clarified by centrifugation at 5000 g for 15 minutes at 4 ^{0}C in a refrigerated centrifuge (Kubota).

5. The Supernatant was collected in 2 ml centrifuge tubes and mixed with equal volume of Chloroform-N-Butanol (1:1 v/v).

6. The content was kept at 4 ^{0}C in refrigerator for 45 minutes with constant Starring.

7. The content was re-centrifuged at 5000 g for 15 minutes at 4 ^{0}C and the aqueous phase was collected in another 2 ml centrifuge tube.

8. To this aqueous phase 4 % PEG (Mw-6000) and 0.2 % NaCl were added and the mixture was kept at 4 ^{0}C in refrigerator with constant starring for 1 hr.

9. This was kept in refrigerated condition over night.

10. Next day the virus precipitate was pelleted at 30000 g for 2 hours.

11. The supernatant was removed carefully and the pallet was resuspended into re-suspension buffer and stored at -80 ^{0}C.

3.2.2.2. Quantification of antigen

3.2.2.2a. Analysis of viral coat protein (antigen) by SDS-PAGE

SDS-PAGE was performed to analyze the coat protein of virus in the total protein isolated from infected and healthy plant samples using the method described by Laemmli (1970) with slight modification. Twelve per cent resolving gel was used for separating the protein based on the molecular weight and 5 per cent stacking gel

was used for aligning the proteins over one another. Detailed procedure was given below.

Reagents used

1. Tris (pH-6.8 and 8.8)
2. 10 % SD
3. 10 % APS
4. TEMED
5. 30 per cent Acrylamide-Bis-acrylamide solution
6. Double autoclaved distilled water
7. Protein loading dye
8. 1X SDS-electrophoresis buffer (running buffer)
9. Stainer solution
10. Developer solution
11. Fixer solution

Chemical composition of buffers, Staining and destaining solutions and protein loading dye are given in **Annexure-III**

Procedure

1. Gel casting frame (clump of two glass plates) was set on the casting stand. Twelve per cent resolving gel and 5 per cent stacking gel was prepared separately (shown in table 2) and poured into the casting plate and finally allowed to solidify at room temperature.

2. After solidification of the gel the comb was removed carefully and the trays were assembled in the SDS-PAGE electrophoresis unit containing 1X running buffer

3. Freshly isolated protein samples were prepared by mixing 10µl of protein with 5µl of gel loading dye.

4. The mixture was heated at 95 ^{0}C for 3 minutes and immediately cooled in ice 20μl of each protein ladder and samples were loaded into the gel.

5. The cathode and anode were connected to power pack and the gel was run at constant voltage of 100 volt for 3 hrs or the samples reaches to the bottom of the plate.

6. The gel was then carefully removed from the casting tray and placed into the fixer solution for 8-10 minutes.

7. After that the gel was washed twice with distilled water and then placed into the staining solution for 8-10 minutes in dark condition. The gel was then washed with distilled water twice and placed into the developer solution for 10-15 minutes for visualizing the band.

8. The gel was removed from the developer solution and placed into the fixer solution for 2-3 minutes.

9. Finally the gel was removed from fixer solution and placed into the distilled water and desired size band was observed.

Table 2: Composition of reagents used for preparation of SDS-PAGE gel

SI. No.	Reagents	Composition	
		For 5 per cent stacking gel	**For 12 per cent resolving gel**
1.	Distilled water	4.1 ml	3.3 ml
2.	4 X Tris (pH-6.8)	0.75 ml	-
3.	4 X Tris (pH-8.8)	-	2.5 ml
4.	Acrylamide Solution	1 ml	4 ml
5.	10 per cent SDS	60 μl	100 μl
6.	TEMED	20 μl	40 μl
7.	10 per cent APS	60 μl	100 μl

3.2.2.2b. Quantification of antigen by Spectrophotometer

The samples which were giving 37 kDa bands in the SDS-PAGE were further analyzed to determine the quality and quantity of the isolated protein using Nanodrop® ND-1000 (Nanodrop technologies Inc., USA). Before taking the reading in the nanodrop the paddle stand of the instrument was wiped properly and then the 1μl of resuspension buffer was used as blank to set the instrument to zero, after that 1 μl of the sample was loaded. The absorbance of the isolated viral protein was measured at a wavelength of OD260/OD280 and OD260/OD230 ratio was observed to determine the purity of the isolated vial protein. A sample whose OD260/OD280 ratio is below 1.8 and OD260/OD230 ratio was below 1 shows the good quality protein without any contamination of DNA and RNA.

3.2.2.3. Development of antiserum against *Cardamom mosaic virus*

3.2.2.3a. Immunization of rabbit

The one year old New Zealand white albino rabbit was used for the production of antibody against CdMV by immunizing the rabbit with viral protein. The rabbit was maintained in the animal house of the Amala cancer research station, Thrissur. The method described by Gonsalves *et al.* (1986) was used for the immunization of rabbit with slight modification. The partially isolated viral protein (5mg/ml) was mixed with freund's complete adjuvant in 1:1 ratio and injected in both the hip muscles. Further injection of the virus was given at weekly interval by mixing the viral protein with freund's incomplete adjuvant in 1:1 ratio. The schedule of immunization of rabbit is presented in table 3.

3.2.2.3b. Collection of blood and Purification of immunoglobulin

The rabbit was immunized with 5mg of antigen and after further four booster doses, from the jugular vein of ear blood samples were collected with the help of sterile syringes and transferred into a sterile eppendorf tube. The eppendorf tubes

containing blood sample were left undisturbed at room temperature until the blood was clotted. The clotted blood was gently cut with the help of sterile toothpick and kept under refrigerated condition over night so that the serum can come out from the clot. After the overnight incubation the clear fluid was collected carefully in eppendorf tube and centrifuged at 6000 g for 5 minutes at 4 ^{0}C. The top straw colored fluid that contains the immunoglobulins was collected in sterile eppendorf tube.

Table 3: Details of injection given to rabbit

SL. No.	Adjuvant mixed with viral protein	Date of Immunization
1	Freund's complete adjuvant	**29/05/2015**
2	Freund's incomplete adjuvant	**13/06/2015**
3	Freund's incomplete adjuvant	**20/06/2015**
4	Freund's incomplete adjuvant	**27/06/2015**
5	Freund's incomplete adjuvant	**12/07/2016**

3.2.2.4. Detection of antibody titre by immune diffusion assay

The basic principle involved in the immuno diffusion assay that the specific antibody will bind with the specific antigen and form an insoluble antigen antibody complex which can be visible with naked eye. The detection of antibody titre was done by using Ouchterlony double diffusion (ODD) assay by the method described by Abida (1995) with some modification. The detailed procedure is described below.

Reagents used

1. ODD-kit (Bangalore Geni)
2. Rabbit antibody (Primary antibody raised against CdMV)

Procedure

100 mg of agarose was dissolved in 10 ml of 1X assay buffer provided in ODD kit by boiling it in microwave oven. 5 ml of the gel solution was then poured on

to the glass plate placed on the horizontal surface and allowed it to set at room temperature. After solidification of the gel, 7 walls were made with the help of puncher and labeled properly. The antibody dilutions of 1:10, 1:100, 1:150, 1:200, 1:250, 1:300 was made by dissolving the desired amount of antibody in 1X assay buffer. 10μl each of viral protein and different dilution of antibody were then loaded in the gel (Figure 1) and kept in the desiccators under humid condition for about 48 hours so that the antibody antigen complex forms precipitation line with different dilution of antibody.

3.2.2.5. Indirect ELISA for the detection of *Cardamom mosaic virus*

Indirect ELISA was carried out to determine the sensitivity of the produced antibody against CdMV and also to determine the serological relation with other viruses. The method described by Koeing in 1981 was used for detection of CdMV through Indirect ELISA with some modifications. The uncoated microtitre plate was coated with crude sap of the infected plant (to be used as antigen) and primary antibody i.e. polyclonal antibody produced against the virus in rabbit was then loaded to bind with viral protein. The secondary antibody conjugated with alkaline phosphatase or HRP (Horse Radish Peroxidase) was loaded to bind with primary antibody and on addition of substrate it develops colour (Figure 2). The detailed procedure is given below.

Reagents used

1. PBS Coating buffer (1X, pH-7.4)
2. Wash buffer (1X, pH-7.4)
3. Blocking buffer (1X, pH-7.4)
4. Antibody dilution buffer
5. Substrate buffer (pH-9.8)
5. Substrate
6. ELISA kit

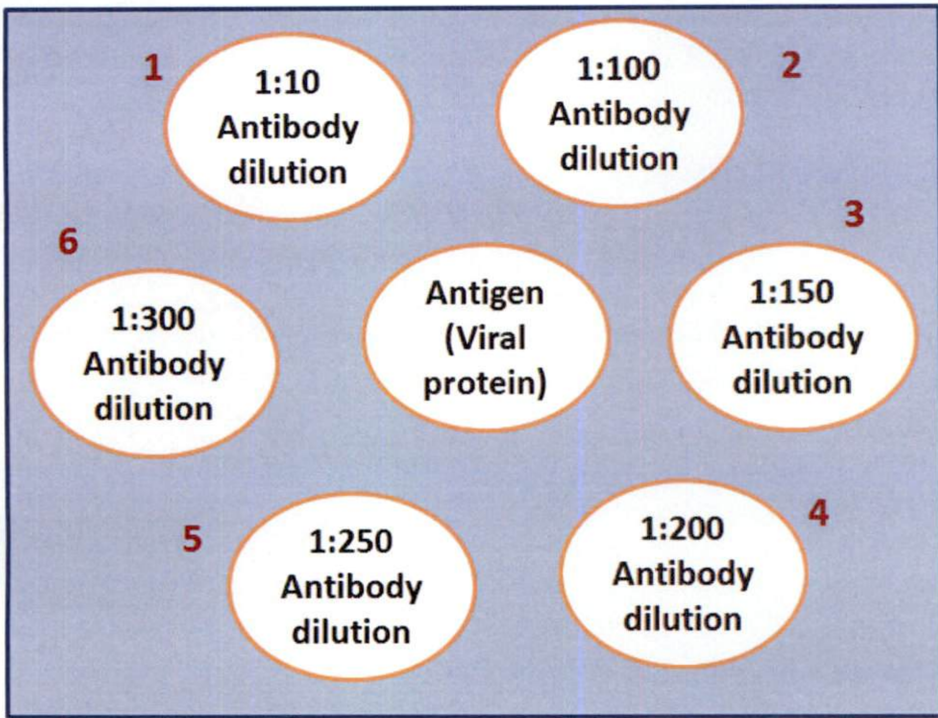


Figure 1: Systematic representation of antigen and different dilution of antibody in Ouchterlony double diffusion (ODD) assay.

Composition of buffers are shown in **Annexure-IV**

Procedure:

1. 1 gm of infected and healthy leaf tissue was pulverized separately in 5 ml of phosphate buffer in pre-chilled motor pestle and transferred in micro centrifuge tubes.

2. The content was centrifuged at 12000g for 15 minutes at 4^{0}C. The supernatant was collected in another sterile micro centrifuge tube and stored in refrigerator.

3. Wells of microtitre plate was then coated with 100 µl of isolated antigen along with control and the plate was incubated at 37 ^{0}C for about 2 hours.

4. The plates were then washed thrice with washing (PBS-T) buffer. After washing the residual site of each wells was then blocked with blocking buffer and incubated for at 37 ^{0}C for 1hr.

5. After that the plates were washed with washing buffer and 100µl primary antibodies (1:10, 1:100 and 1:150 dilutions) was loaded into the wells and incubated at 4^{0}C for overnight.

6. After overnight incubation the plates were washed thrice with washing buffer to remove the unbounded primary antibodies from the wells. 100 µl of secondary antibodies was then added into each well and incubate for 37 ^{0}C for 2 hours.

7. After the incubation, the plates were again washed thrice with washing buffer to remove the unbounded secondary antibody and then 100µl of substrate (pPNP or TME provided in ELISA kit) was added and incubated in dark at room temperature for about 30 minutes for development of colour.

8. Finally the plates were observed visually for differentiation between healthy and infected samples based on their colour intensity in the wells and later the

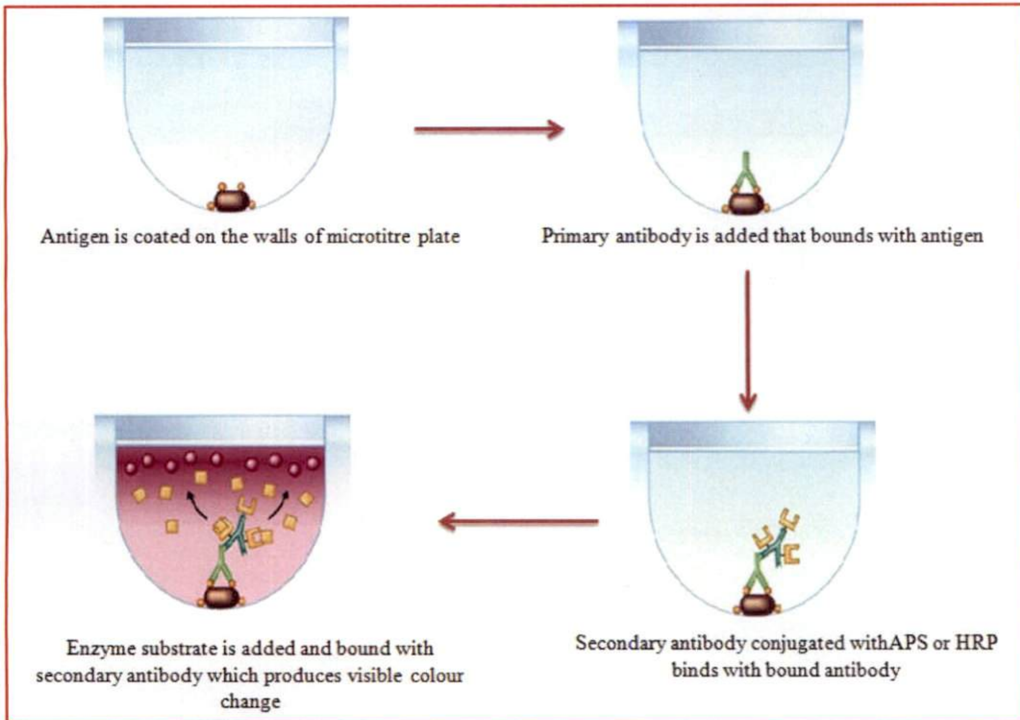


Figure 2: Systematic representation of indirect ELISA for the detection of viruses

colour absorbance was taken in the ELISA plate reader at 405 nm for final confirmation.

3.2.3. Detection of *Cardamom mosaic virus* by Reverse Transcriptase (RT)-PCR

3.2.3.1. Isolation of total RNA

Isolation of good quality and quantity of RNA from infected and healthy leaf of cardamom is the pre requisite for the RT-PCR analysis. The total RNA was using Trizol method (Sigma-Aldrich, USA). The detailed procedure for the RNA isolation is given below.

Reagents used

1. Trizol-reagent
2. Chloroform
3. Ice cold Isopropanol
4. PVP
5. β-mercaptoethanol
6. 75 per cent ethanol
7. Autoclave DEPC treated water

Procedure

1. 100 mg of leaf was pulverized well in liquid nitrogen using DEPC treated motar and pestle.
2. one ml of Trizol was added and the homogenate was incubated at room temperature till the solution become brown.
3. Homogenate was then transferred into a 2 ml centrifuge tube.
4. Content was centrifuged at 12000g for 10 minutes at 4^0C.
5. The supernatant was transferred to a 2 ml centrifuge tube.

6. 200µl of ice cold chloroform was added to the supernatant and the content was shacked vigorously for 15 second and then kept at room temperature for 10 min.

7. The content was than centrifuged at 12000g for 10 minutes at 4^0C.

8. The supernatant was transferred into 1.5 ml micro centrifuge tube and 500 µl of ice cold isopropanol was added.

9. The contents was mixed by slight inversion and incubated in ice for 10 min and then centrifuge at 12000g for10 minutes at 4^0C.

10. The supernatant was discarded and pellet was washed with 1 ml of 75% ethanol by centrifuging at 5000g for 2 minutes at 4^0C.

11. The supernatant was discarded and the pellet was dried under laminar air flow.

12. The pellet was dissolved in 25µl of DEPC treated water.

3.2.3.2. Quality and quantity of analysis of total RNA

3.2.3.2a. Quality analysis by Formaldehyde-agarose gel electrophoresis

The 1 per cent formaldehyde agarose gel electrophoresis was performed to check the quality of total RNA by using the protocol given by Babaurao (2012).

Materials used for gel electrophoresis

1. Agarose
2. 10X MOPS (pH-7)
3. 6X gel Loading/tracking dye
4. Ethidium bromide solution (0.5ug/ml)
5. UV-Transilluminator
6. Gel Doc

Chemical composition of buffers and gel loading dye are given in **Annexure-V**

Procedure

One liter 1X MOPS buffer was prepared by diluting 100 ml of 10X MOPS buffer in 900 ml of double autoclaved DEPC treated water. This buffer was used for the preparation of gel and filling the electrophoresis tank. The open end of casting tray was sealed with cello tape and kept on a horizontal platform and comb was placed at the one end. 0.5 gm of agar was dissolved in 47.3 ml of 1X MOPS buffer by boiling in microwave oven and allowed to cool for few minutes at room temperature. After that 3µl of ethidium bromide and 2.7 ml of formaldehyde was added and poured into the tray. The gel was allowed to solidify at room temperature. After the solidification of gel, the cello tap and the comb were removed and the gel was kept in the electrophoresis tank containing 1X MOPS buffer (with the wells facing towards the cathode) in such a way that it submerged to a depth of 1 cm. 5µl of total was taken and incubated in PCR at 95 ^{0}C for 3 minutes for denaturation and immediately cooled on ice. Denatured RNA was mixed with 1µl of 6X loading dye and loaded in the gel along with 3µl of DNA ladder. The cathode and anode were connected to power pack and the gel was run at 50 volt for about 2 hours. The power was turned off when the tracking dye moved 5 cm from the wells. The gel was then placed in the gel doc were bands were visualized under UV-Light and gel image was documented.

3.2.3.2b. Spectrophotometric analysis of total RNA

The samples which were giving 3 intact bands in the formaldehyde agarose gel were further analyzed to determine the quality and quantity of the isolated total RNA using Nanodrop® ND-1000 (Nanodrop technologies Inc., USA). Before taking the reading in the nanodrop the paddle stand of the instrument was wiped properly and then 1µl of double autoclaved DEPC treated water was used as blank to set the instrument to zero, after that 1µl of the sample was loaded. The absorbance of the total RNA was measured at a wavelength of OD260/OD280 and OD260/OD230 ratio was observed to determine the purity of the isolated total RNA. A sample whose

OD260/OD280 ratio comes in between 1.8 to 2.0 and OD260/OD230 ratio comes above 1 show the good quality RNA without any contamination of DNA and protein.

3.2.3.3. Synthesis of cDNA

The total RNA isolated from the infected and healthy leaf of the cardamom was converted into the cDNA for the synthesis of first strand. The cDNA kit provided by thermo fisher was used for the synthesis of the first strand of the DNA by using oligodt-primer provided in the kit. The detailed procedure for RNA to cDNA conversion is given below.

Reagents used

1. Revert aid H minus First strand synthesis kit
2. Chloroform (95%)
3. Isopropanol (95%)
4. Ethanol (75%)
5. DEPC treated- double autoclaved distilled water

Procedure

1. Three µl of total RNA (1µg), 1 1µl of oligodT primer and 8µl was added to a 0.2 ml of micro centrifuge tubes.

2. The mixture was incubated in at 65^{0}C for 5 min in thermo cycler and then quick chilled on ice. The mixture was then spanned briefly to collect the content at the bottom of the tube.

3. Four µl of 5X first strand buffer, 2µl of 10mM dNTPs, 1µl RNase OUT recombinant ribonuclease inhibitor and 1 RNase OUT recombinant ribonuclease inhibitor, 1 µl M-MuLV reverse transcriptase of were added one by one in the tube.

4. The content was mixed gently and incubated at 42^0C for 10 minutes.

5. The reaction was inactivated by heating content at 70^0C for 5 minutes and stored at 80^0C.

3.2.3.4. Primer designing and validation.

Primer pairs for CdMV were designed based on the conserved region in the coat protein using primer3 software (http://primer3.ut.ee/). The coat protein sequence for *Katte mosaic virus* from different location were downloaded from the NCBI data base (Shown in table 4) and fed into the primer3 software for development of primers sequence. The primer sequences generated by primer3 software were then validated for its heterodimer formation, hair pin loop structures and Tm mismatch by using oligodt analyzer software (https://eu.idtdna.com/calc/analyzer). After validation the primers sequences were send to Scigenome, Kochi for development of primer.

Table 4: Geographical origin and gene bank accession number of *Cardamom mosaic virus* isolates used for primer designing.

Sl. No.	Region	State	Accession no.
1	Pampadumpara, Idukki	Kerala	JN544077
2	Meppadi, Wayanad	Kerala	JN544080
3	Kattappana, Idukki	Kerala	AJ312774
4	Vandiperiyar, Idukki	Kerala	AJ308477
5	Kursupara, Idukki	Kerala	AJ30846
6	Sakleshpur, Hassan	Karnataka	JN544082
7	Thadiyankudisai, Dindigul	Tamil nadu	JN544078
8	Madikeri, Kodagu	Karnataka	AJ308474

3.2.3.5. Synthesis of second strand

The synthesis of second strand of cDNA was carried out in the PCR (Thermo cycler) by using the primers developed by primer3 software. All developed primers were used for the amplification of the coat protein gene for the characterizations of CdMV.

Reagents used

1. 10X reaction buffer
2. dNTP mix (2.5 mM)
3. $MgCl_2$
4. Forward and Reverse primer
5. Taq DNA polymerase
6. Nuclease free water
7. cDNA

Procedure

Master mix prepared for the PCR reaction as indicated in table 5a and the content was spun briefly for proper mixing. After that the aliquot of the master mix was added to each special thin walled tubes of uniform thickness to ensure the equal change in the temperature throughout the PCR reaction and finally the cDNA template was added separately. The PCR reaction was standardized for the second strand synthesis as shown in table 5b.

3.2.3.6. Agarose gel analysis for identification of virus specific amplicon

After the synthesis of the second strand, the PCR product was electrophoresed in 1 per cent agarose gel for the identification of virus specific amplicon. The detailed procedure is given below.

Table 5a: Composition of reaction mixture for PCR

S.No.	Components	Volume per reaction (μl)
1	Nuclease free water	12
2	10X reaction buffer	2.5
3	$MgCl_2$ (25 mM)	2
4	dNTP mix (2.5 mM)	2
5	Forward primer	0.5
6	Reverse primer	0.5
7	Taq DNA polymerase	5
8	cDNA	2
9	**Total**	**25**

Table 5b: Temperature profile for second cDNA synthesis

Sl. No.	Step	Temperature	Time
1	Initial denaturation	94 ^{0}C	2 min
2	Denaturation	94 ^{0}C	30 second
3	Annealing	45-60 ^{0}C	45 second or 1 min
4	Extension	72 ^{0}C	1 min
5	Step 2 to 4	35 cycles	-
6	Final extension	72 ^{0}C	10 min
7	Storage	4 ^{0}C	Infinity

Reagents used

1. Agarose
2. 50X TAE buffer (pH-8)
3. Gel loading dye
4. DNA ladder
5. Ethidium bromide
6. 70% Ethanol
7. PCR- product

Chemical composition of buffers and dye is shown in **Annexure-V**

Procedure

The frame of the gel casting tray was cleaned with 70 per cent ethanol and open sides of the tray was sealed with cello tap. The frame was then kept on the flat surface. The comb was placed parallel to the open edge of the frame about 3mm above the surface of the tray. The desired amount of agarose powder was dissolved in 1X TAE buffer by boiling the content at 100 ^{0}C in a microwave oven and cooled to 50^{0}C. About 5 μl of ethidium bromide (0.1g in 10ml of water) was then added to the gel and poured into the gel frame and allowed to set at room temperature. After setting, the gel was transferred to the electrophoresis tank in such a way that the walls face towards the anode. The tank was then filled with running (1X TAE) buffer just enough to cover the gel. 25 μl of the PCR product was mixed with 2 μl of 6X gel loading dye and loaded in the wells of submerged gel along with the 3 μl of DNA(100 bp) ladder using a micropipette. The electrophoresis unit was connected to the power supply and electrophoresis was carried out at 70 volt for 2 hr or up to deep blue dye reaches the end of the gel. It was then visualized and documented by Gel doc XR.

3.2.3.5. Elution of virus specific amplicon and sequencing

The gel piece containing virus specific amplified DNA fragment was cut from the agarose gel under the UV-transilluminator by using sterile blade. The DNA fragment was then separated from the gel by using Elution kit (Cat no.- 63458) purchased from Sigma Aldrich.

The eluted virus specific DNA fragment was again reamplified using the PCR protocol as mention above and directly sequenced using the automated sequencing facility available at Scigenome, Kochi.

3.2.3.5. *In-silico* analysis of sequenced product

The sequences obtained from sequencing were annoted using bioinformatics tools. For the sequence comparison, the published nucleotide and amino acid sequences from different isolates of *Katte mosaic virus*, other members of potyviruses were used. The Nucleotide similarity search was done by using BLASTn and BLASTx (Altschul *et al.*, 1990).

The phylogenetic analysis was performed to determine the variability among the isolates collected from different location of Kerala. The phylogenetic tree was drawn with help of software MEGA.7 using neighbor joining method (Balasubramanian and Selvarajan, 2014).

Results

4. Results

The katte disease of cardamom is one of the most destructive diseases of cardamom caused by *Katte mosaic virus* or *Cardamom mosaic virus*. The results of different experiments carried out for "Molecular characterization of *Katte mosaic virus* in cardamom (*Elattaria cardamomum* Melton)" at the centre for plant Biotechnology and Molecular Biology (CPBMB) during 2014-2016 are presented in this chapter. The silent findings of the study are presented below.

4.1. Collection and maintenance of healthy and infected plant samples

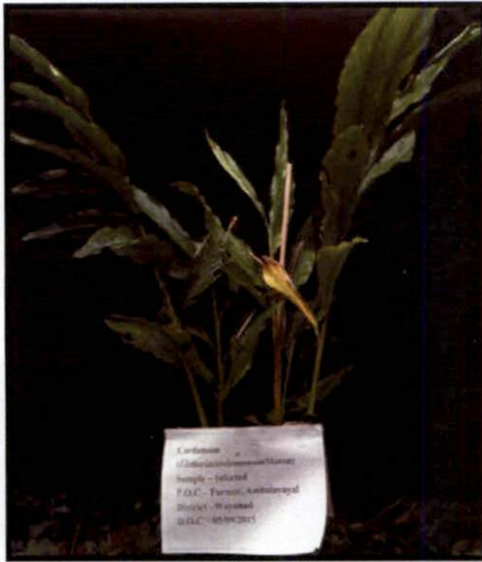
The katte infected and healthy plants of Njallani green gold variety of cardamom were collected from different locations of Wayanad and Idukki districts of Kerala based on the symptoms produced on the plant (Plate 1-3). The plants showing mosaic symptom on leaf and pseudo stem with chlorotic fleck (Plate 4) were collected and planted in the field under insect proof net. The infected and healthy plants were planted with adequate isolation distance.

4.2. Detection of *Cardamom mosaic virus* by ELISA

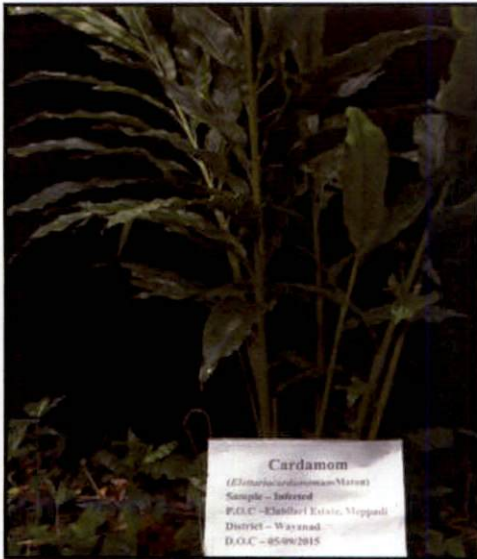
4.2.1. Partial purification of *Cardamom mosaic virus* from cardamom

Efforts had been made to isolate the coat protein of *Cardamom mosaic virus* from the infected leaves of cardamom which is the primary host for CdMV. The young leaves showing prominent mosaic symptoms with chlorotic flecks were collected from different locations of Wayanad and Idukki districts Kerala which were used for the study. The modified method of Gonsalves *et al.* (1986) was tried for the partial purification of the coat protein from the infected leaf samples, as the virus has been grouped under potyvirus genus.

In the present study, the protein was purified from other contaminants using Chloroform: n-Butanol mixture and the virus were precipitated by using poly



A



B

Plate 1: Infected samples from different locations of Wayanad district of Kerala (A- Ambalavayal; B- Meppadi)

A

B

C

D

Plate 2: Infected samples from different locations of Idukki district of Kerala

(A- Paravalam; B- Irettyar; C- Pampadumpara; D- Myladumpara)

A **B**

Plate 3: Healthy samples collected from different location of Kerala

A- Ambalavayal (Wayanad district); B- CRS, Pampadumpara (Idukki district)

A) Mosaic with chlorotic flack symptom on the leaf

B) Mosaic symptom on pseudostem

Plate- 4: Symptom produced by katte mosaic virus on cardamom leaf and

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ethylene glycol (PEG Mw-6000) with high speed centrifugation to prevent the aggregation and loss of virus during low speed centrifugation. The particle aggregation of virus was prevented by using urea in the resuspension buffer and extraction buffer. The coat protein obtained by this method contains low amount of host protein.

4.2.2. Quantification of antigen

4.2.2a. Analysis of protein by SDS-PAGE

The partially purified protein was analyzed by SDS-PAGE. This is the most commonly used method for analyzing the presence of coat protein of the virus in the isolated protein samples by characterizing the virus based on their molecular weight.

The analysis of isolated protein from infected leaf of cardamom was resolved in 12 per cent separating gel, revealed the presence of a band with the approximate molecular weight of 37 kDa was observed in the six infected samples of cardamom which is the expected coat protein size of the CdMV and also absence of such bands in the healthy protein samples confirms the infection (Plate 5).

4.2.2b. Quantity and quality analysis of total protein

The Spectrophotometric analysis of the isolated protein was carried out to know the quality and quantity with the help of the nanodrop. The absorbance at $A_{260/280}$ was documented. The result shows that samples are having 4.98 to 9.20 mg per ml of protein with OD for $A_{260/280}$ ranging from 1.30 to 1.44 indicating that the isolated protein has good quantity of protein with less contamination of DNA and RNA (Table-6).

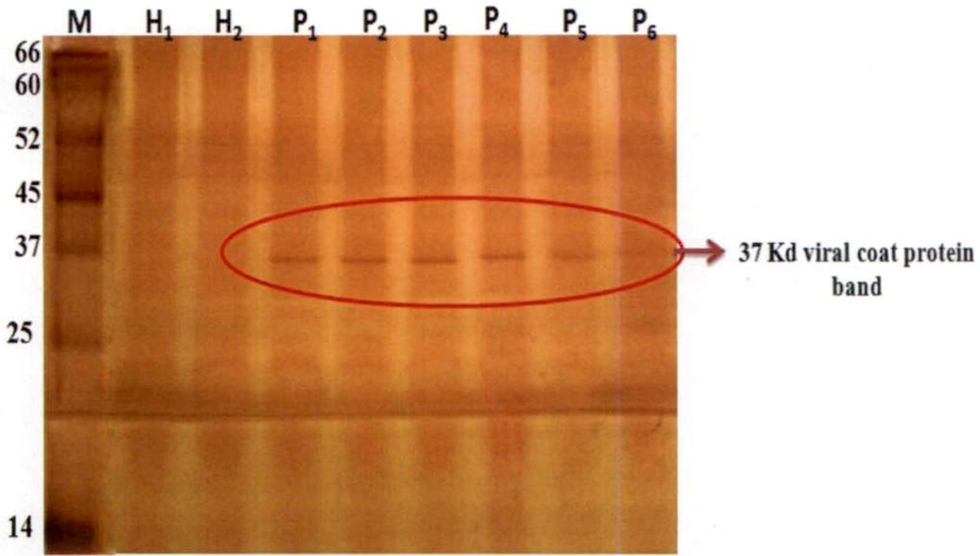

Plate 5: SDS-PAGE analysis of partially purified *Katte mosaic virus* coat protein

M- Protein ladder (14-66 KDa); H_1 and H_2 – Healthy samples

P_1 to P_6- Infected samples from different locations

P1- Ambalavayal; P2- Meppadi; P3- Paravalam; P4- Irettyar

P5- Pampadumpara; P6- Myladumpara

Table 6: Quality and quantity analysis of total purified protein using nanodrop spectrophotometer.

Name of sample	Quantity of Protein(mg/ml)	OD 260/280
H1-Healthy	6.80	1.44
P1- Ambalavayal	9.20	1.32
P2- Meppadi	8.68	1.35
P3- Paravalam	8.26	1.40
P4- Kattapana	8.88	1.48
P5- Pampadumpara	5.80	1.30
P6- Myladumpara	4.98	1.33

(P – Infected plant samples)

4.2.3. Detection of antibody titre by immuno diffusion assay

The ODD (Ouchterlony Double Diffusion) assay was carried out to determine the antibody titre. The different dilutions of primary antibody produced in the rabbit were loaded in the wells along with coat protein of the virus to form an antigen and antibody complex (Table 7). The result of the ODD revealed that the 1:10,1:100 and 1:150 dilution of antiserum forms the insoluble antigen and antibody complex with coat protein of *Cardamom mosaic virus* in ODD assay (Plate 6).

Table 7: Determination of antibody titre with different dilutions of primary antibody in ODD assay.

SL. No.	Antibody dilution used	Appearance of Band (Antigen antibody complex)
1	1:10	Yes
2	1:100	Yes
3	1:150	Yes
4	1:200	No
5	1:250	No
6	1:300	No

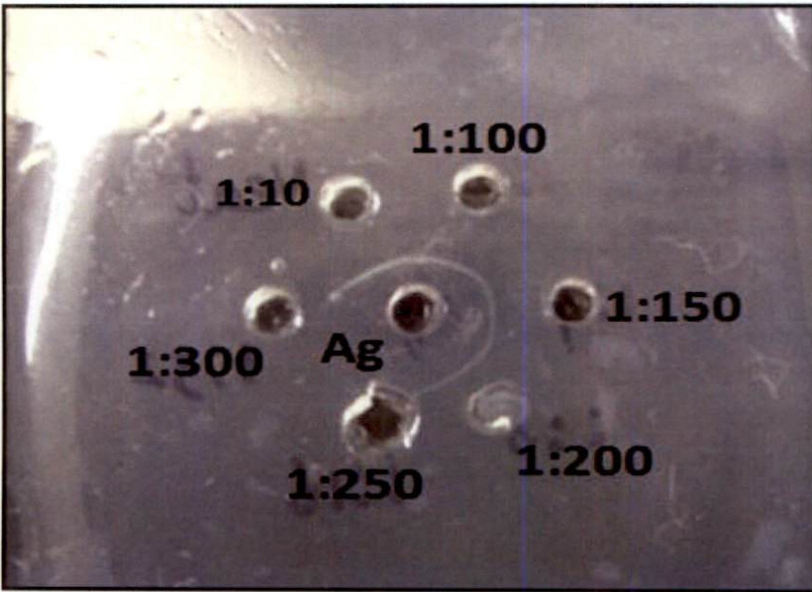

Plate 6: ODD gel profile with different dilutions of antibody

4.2.4. Detection of *Cardamom mosaic virus* using Indirect ELISA

Indirect ELISA was performed by using polyclonal antisera raised against CdMV in rabbit to detect the virus in the infected leaf extract of cardamom plants. In order to detect the CdMV, indirect ELISA was carried out with different concentration of antisera (1:10, 1:100 and 1:150 dilutions) which were found through ODD assay with 1:200 dilutions of secondary antibody. The absorbance at 405nm was recorded using ELISA reader. The result obtained 30 minute after adding the substrate buffer are shown in Table 8 and 9.

The result of indirect ELISA shown that the CdMV can be easily detected in the infected leaf extracts by using 1:100 dilution of primary antibody raised against CdMV in rabbit with 1:200 dilution of antirabbit secondary antibody conjugated with alkaline phosphatase (Plate 7a) or horse radish Peroxidase (Plate 7b).

7. Serological relationship of *Cardamom mosaic virus* with other viruses

In order to get the serological relationship of the CdMV with other viruses, Indirect ELISA was carried out with the polyclonal antibody of the BBrM (*Banana bract mosaic virus*), BBTV (*Banana bunchy top virus*) and CMV (*Cucumber mosaic virus*) purchased from AGDIA, USA. Leaf extracts of infected samples was used as antigen in indirect ELISA. After 30 minute of adding the substrate absorbance at 405 nm was documented by using ELISA reader (Table 10).

Based on the absorbance value the reaction was called as positive when the absorbance value of infected > 2 times of the healthy samples (Smitha, 2004).

The results of indirect ELISA has revealed that the crude sap of katte infected cardamom leaf tissue had cross reacted with the BBrM antiserum (Plate 8). The results clearly indicated that *Cardamom mosaic virus* shows some similarity with the BBrM virus because both belong to family potyviridae.

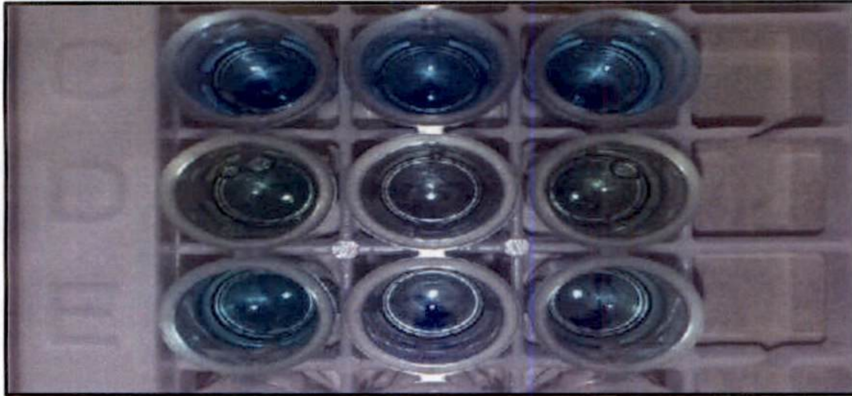

Plate 7a: Detection of *Cardamom mosaic virus* using Indirect ELISA through HRP conjugated secondary antibody.
(C- Blank; D- Infected samples; E- Healthy samples)

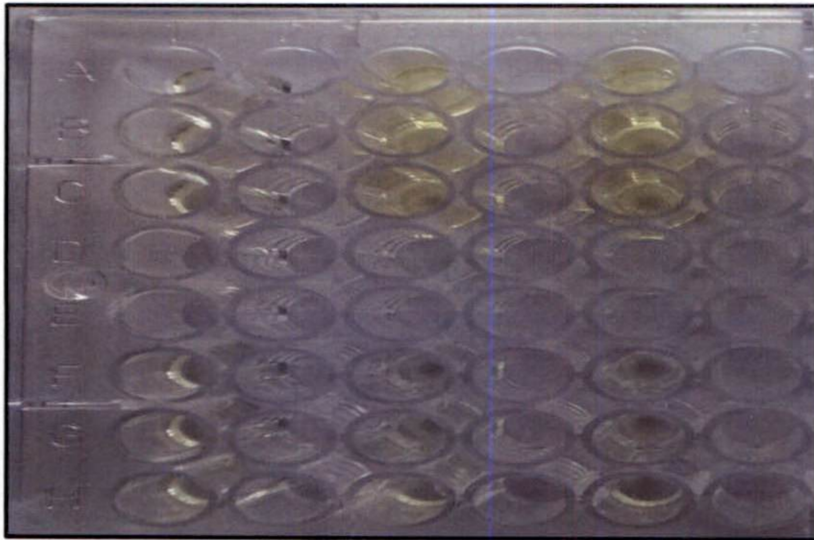

Plate 7b: Detection of Cardamom mosaic virus using indirect ELISA with APS conjugated secondary antibody.

1 (A, B, C) - Infected samples with 1:10 dilution of antibody

3 (A, B, C) - Infected samples with 1:100 dilution of antibody

5 (A, B, C) – Infected samples with 1:150 dilution of antibody

1 (F, G, H) – Control

3 (F, G, H) - Healthy

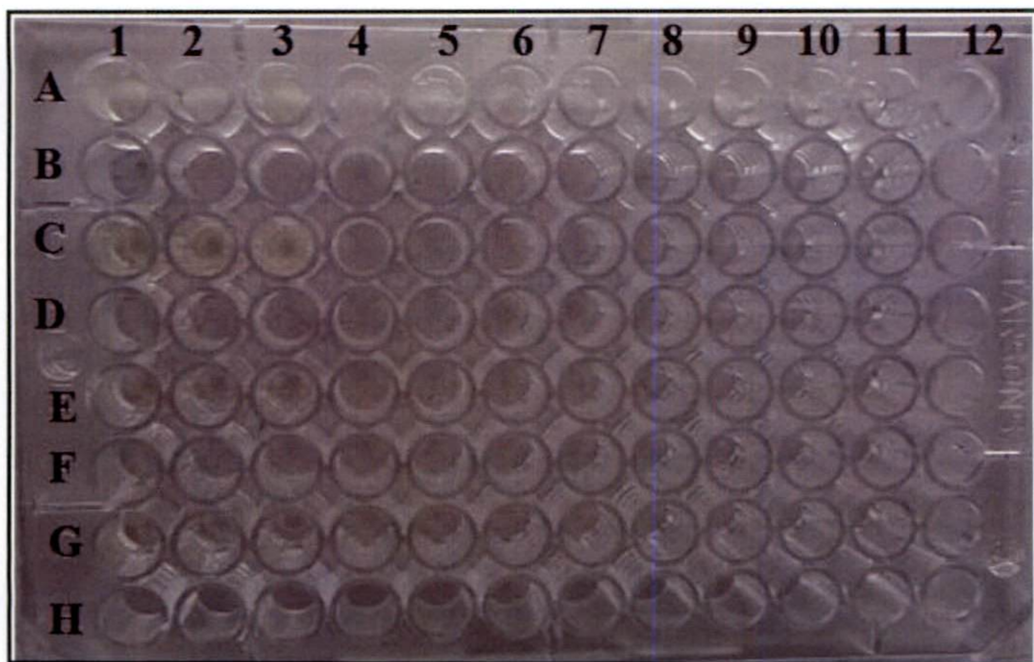

Plate 8: Detection of Serological relationship of katte mosaic virus with other viruses using indirect ELISA

A (1, 2, 3)- Healthy sample with BBrMV antibody

A (5, 6, 7)- Healthy sample with BBTV antibody

A (9, 10, 11)- Healthy sample with CMV antibody

C (1, 2, 3)- Infected samples with BBrMV antibody

F (1, 2, 3)- Infected sample with BBTV antibody

G (1, 2, 3)- Infected sample with CMV antibody

Table 8: Analysis of cardamom plant samples using ELISA reader with HRP conjugated secondary antibody.

Dilution of Antiserum	Absorbance at 405 nm		
	Control	Healthy	Infected
1:10	0.101	0.192	0.468
1:100	0.100	0.134	0.659
1:150	0.110	0.183	0.387

Table 9: Analysis of cardamom plant samples using ELISA reader with APS conjugated secondary antibody.

Dilution of Antiserum	Absorbance at 405 nm				
	Control	Healthy	Infected		
1:10	0.110	0.139	0.366	0.344	0.319
1:100	0.105	0.133	0.708	0.688	0.705
1:150	0.112	0.135	0.296	0.298	0.266

Table 10: Analysis of katte disease sample with different polyclonal antibodies raised against viruses from poty and other group using ELISA reader for determining the serological relationship.

SL. No.	Antisera used	Absorbance 405 nm					
		Healthy			Infected		
1	BBrMV	0.317	0.262	0.364	0.916	0.958	0.976
2	CMV	0.140	0.130	0.133	0.147	0.151	0.137
3	BBTV	0.146	0.155	0.169	0.161	0.154	0.159

4.3. Detection of *Katte mosaic virus* through Reverse transcriptase (RT)-PCR

4.3.1. Isolation of total RNA from healthy and infected leaf samples

Total RNA from the infected and healthy samples was isolated using trizol method. The isolated samples were run in the 1 per cent formaldehyde agarose gel in 1X MOPS buffer. The samples having three intact bands indicating 28S, 18S, and 5S + tRNA were of good quality and used for the C-DNA synthesis (Plate-9). The total RNA isolated from the samples was also quantified in the Nanodrop. The ratio of OD260/280 for the samples was greater than 1.8 showing the good quality of RNA without contamination with DNA and Protein (Table 11).

Table 11: Quality and quantity analysis of total RNA using Nanodrop

Name of sample	Quantity of RNA(µg/ml)	Value of OD260/280
H1- Healthy samples	1517.4	2.001
P1- Ambalavayal	1967	1.980
P2- Meppadi	1223.2	2.028
P3- Paravalam	901.58	1.907
P4- Irettyar	996.58	1.977
P5- Pampadumpara	591.87	1.907
P6- Myladumpara	842.41	1.949

(P- Infected plant samples)

4.3.2. Synthesis of cDNA from total RNA

The total RNA was converted into cDNA by using H minus First strand cDNA synthesis kit provided by thermo scientific. The cDNA was synthesized by using M-MuLV reverse transcriptase and oligo primer. The RNA strand was removed by RNase inhibitor and reaction was terminated at 70^{0}C for 5 minutes. The

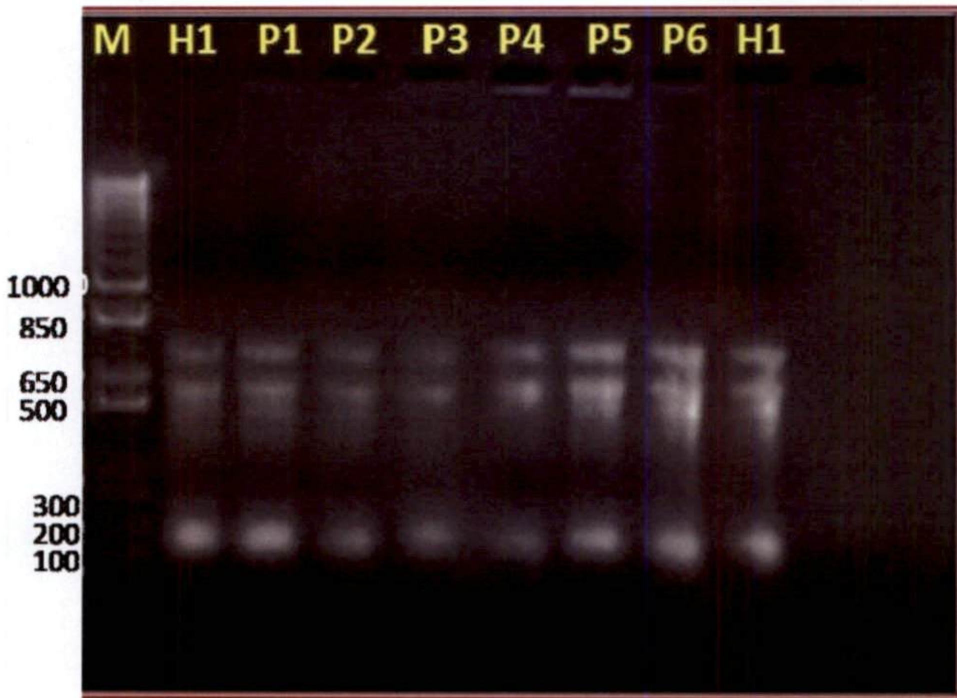

Plate 9: The gel profile obtained by Formaldehyde agarose analysis of total RNA isolated from the healthy and infected samples.

H_1- Healthy; P1- Ambalavayal; P2- Meppadi; P3- Paravalam; P4- Irettyar P5- Pampadumpara; P6- Myladumpara; M- Marker (1 Kb plus)

conformation of the cDNA was done by using the actin primer and PCR protocol provided in the kit was used to synthesize cDNA. The amplified product was analyzed in 1 per cent agarose gel and presence of 450 bp band in agarose gel electrophoresis confirms the synthesis of cDNA from total RNA (Plate 10).

4.3.3. Primer designing and validation

The primers targeting the coat protein regions were designed by using the primer3 soft ware. The coat protein sequence for the CdMV were downloaded from NCBI data base (Figure 3) and submitted to primer3 software for development of primer (Figure 4 and 5). The details of the primer developed by primer 3 software are presented in Table 12. The primer sequence developed by primer 3 software was then validated by using Oligodt analyzer software (Figure 6) to check parameters such as hertrodimer, homodimer, GC content, hairpin structure and Tm mismatch which is required for the good amplification of target gene. The results of oligo dt analyzer shows that the primer developed by primer 3 software can be used for the amplification of coat protein region of the CdMV without any heterodimer and homodimer formation (Table 13).

4.3.4. Determination of annealing temperature

The gradient PCR were performed to determine the annealing temperature of the different primer pairs designed by using primer3 soft ware. The gradient PCR was performed at 15 different temperatures (45-60^{0}C) and annealing temperature for different primer pairs were identified and presented in Table 14.

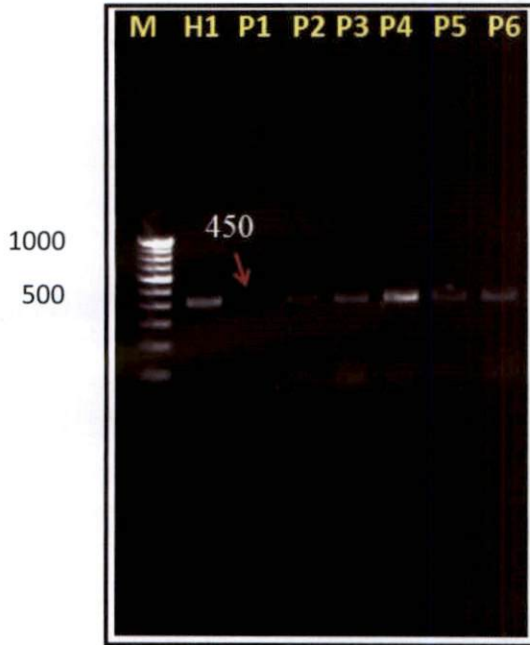

Plate 10: Gel profile of cDNA amplified with actin primers

P1- Ambalavayal (Infected) **P5- Pampadumpara (Infected)**

P2- Meppadi (Infected) **P6- Myladumpara (Infected)**

P3- Paravalam (Infected) **H1- Healthy samples**

P4- Irettyar (Infected)

Figure 3: Retrieval of sequence related to Cardamom mosic virus from NBCI database

Figure 4: Submission of sequence to Primer3 software

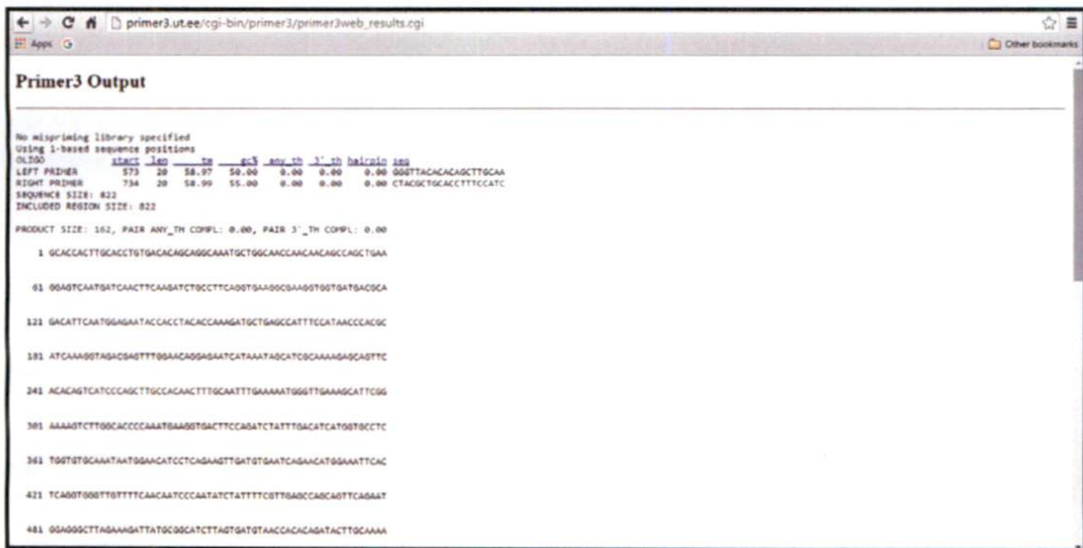

Figure 5: Result page of Primer3 software

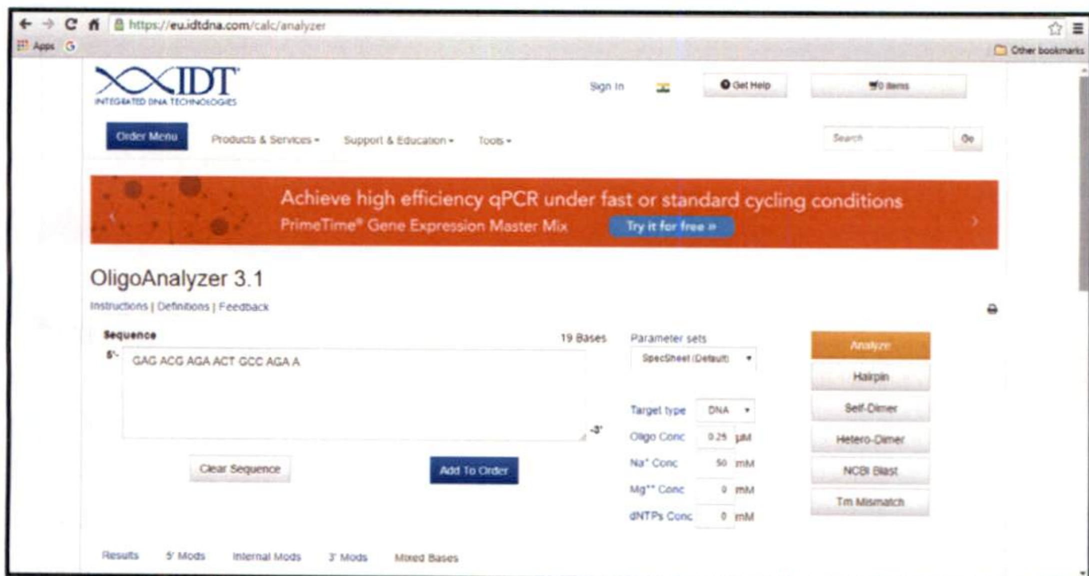

Figure 6: Validating the primers using IDT OligoAnalyzer3.1 software

Table 12: Details of DNA primers developed using primer3 Software

S.no	Accession No.	Name of primer	Region	Forward Primer	Reverse Primer	Expected Band size
1	JN544077	CP-1	Pampadumpara, Kerala	AGCCACTTCCATAACCCACG	CACCAGAGGCACCATGATGT	250
2	JN544080	CP-2	Meppadi, Kerala	GCCATTTCCATAACCCACGC	CACCAGAGGCACCATGATGT	250
3	AJ312774	CP-3	Kattappana, Kerala	CTCGCCACAACACTGCAATT	CCCCATTCTGAACTGCTGGT	250
4	AJ308477	CP-4	Vandiperiyar, Kerala	CAACTCGCCACAACAATGCA	GCCCTCCATTCTGAACTGCT	250
5	AJ30846	CP-5	Kursupara, Kerala	GAGAATACCACCCACGCCAA	CACCAGAGGCACCATGATGT	250
6	JN544082	CP-6	Sakleshpur, Karnataka	GGGTTACACACAGCTTGCAA	TTGCTTCGCTGAACTTTCCC	250
7	JN544078	CP-7	Thadiyankudisai, Tamil Nadu	AGGTGGTGATGATGCAGACA	ATCGCCTTCATTTGGAGTGC	250
8	AJ308474	CP-8	Madikeri, Karnataka	AGACGGGAAAGTACAGCGAA	TGAGGGTTACTAGGCGTTGG	250
9	JN544077	CP-9	Kerala	CTTGCACCAATGACGGAACA	GTGTGATTCAGCAGCAGGTC	800
10	JN544080	CP-10	Kerala	TGTGACACAGCAGGCAAATG	CTACGCTGCACCTTTCCATC	700
11	AJ308477	CP-11	Kerala	GGAGAATACCACCCACACCA	AACTTGTTCTACTGCGCTGC	600

Table 13: Details of primer validation using oligo analyzer 3.1 software

S.no	Name of primer	Homodimer (Maximum 5bps are allowed)	Heterodimer (Maximum 5bps are allowed)	Tm Mismatch	GC content (in per cent)		Hairpin loop (Maximum 3 loop are allowed)	
					Forward	Reverse	Forward	Reverse
1	CP-1	3	2	Weak	57	55	1	1
2	CP-2	2	1	Weak	55	55	2	1
3	CP-3	2	2	Weak	50	55	3	1
4	CP-4	1	2	Weak	50	55	2	1
5	CP-5	3	2	Moderate	55	55	1	2
6	CP-6	4	2	Moderate	50	50	1	1
7	CP-7	2	3	Weak	50	50	1	1
8	CP-8	3	2	Moderate	50	55	2	1
9	CP-9	1	2	Weak	50	55	1	1
10	CP-10	4	2	Weak	50	55	1	2
11	CP-11	4	3	Weak	50	55	1	2

Table 14: Annealing temperature of primers

SL. No.	Primer name	Annealing temperature (in ^{0}C)
1	CP-1	52.5
2	CP-2	52.5
3	CP-3	52.5
4	CP-4	54.5
5	CP-5	53.5
6	CP-6	54.5
7	CP-7	53.5
8	CP-8	53.5
9	CP-9	47.5
10	CP-10	50.3
11	CP-11	51.3

4.3.5. Characterization of *Cardamom mosaic virus* using coat protein specific primers

The second strand of cDNA was synthesized by using the primers targeting the coat protein region of the virus. Each primer pairs were subjected to PCR amplification for amplifying of coat protein region of the virus.

The results of PCR amplification revealed that out of 11 primer pairs, 8 pairs of primer were able to amplify the coat protein region of the virus (Table 15). The primers such as CP-1, CP-2, CP-4, CP-5 and CP-7 were amplifying the coat protein gene and showed 220 base pairs band in 1percent agarose gel (Plate 11-15). Whereas, CP- 9 showed 950 bp, CP-10 showed 750 bp and CP-11 showed 650 bp bands in 1 per cent agarose gel (Plate 16-18). Such bands were present only in the infected samples but absent in healthy samples. This shows that these primers can be

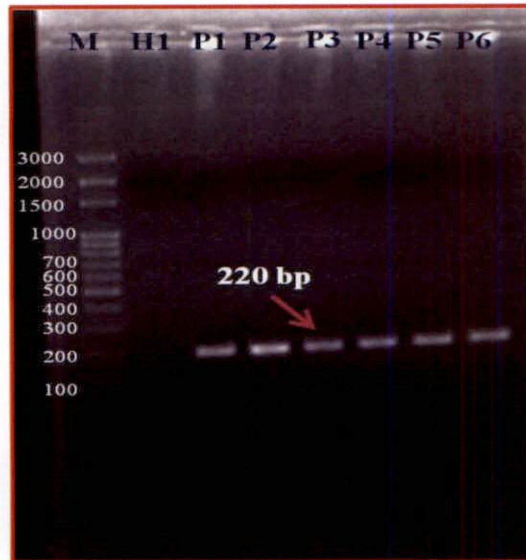

Plate 11: **Characterization of *Cardamom mosaic virus* with CP-1**

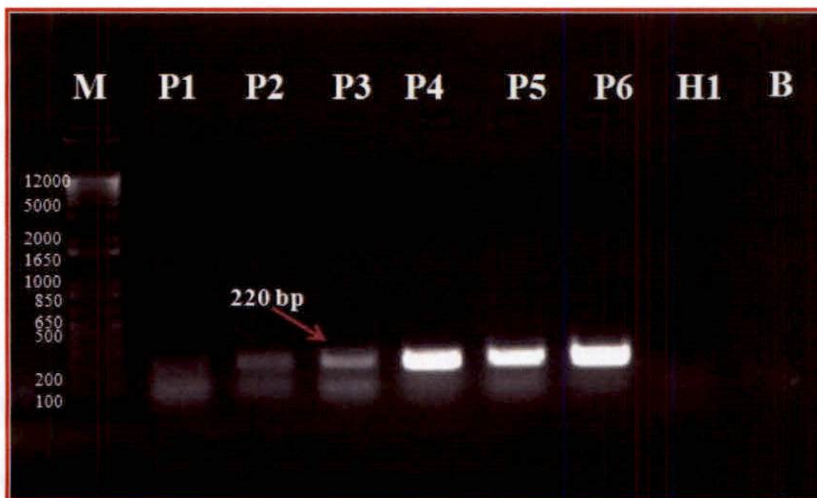

Plate-12: Characterization of *Cardamom mosaic virus* with CP-2

P1- Ambalavayal (Infected) **P5- Pampadumpara (Infected)**

P2- Meppadi (Infected) **P6- Myladumpara (Infected)**

P3- Paravalam (Infected) **H1- Healthy samples**

P4- Irettyar (Infected) **B- Blank** **M- Ladder (1Kb plus or 100bp plus)**

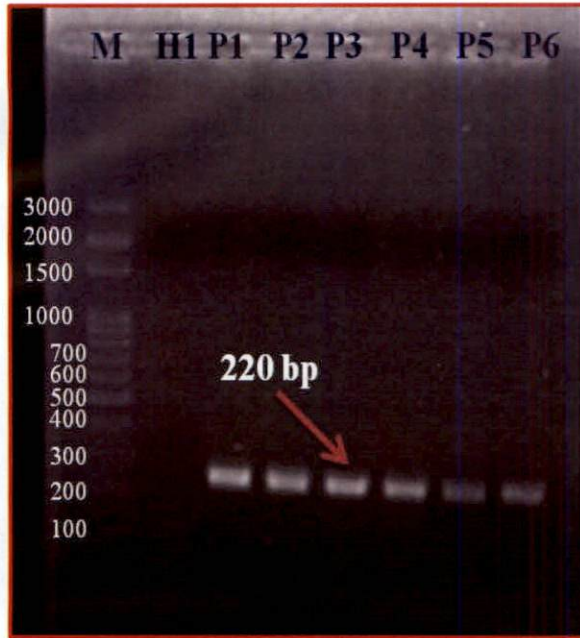

Plate 13: Characterization of *Cardamom mosaic virus* with CP- 4

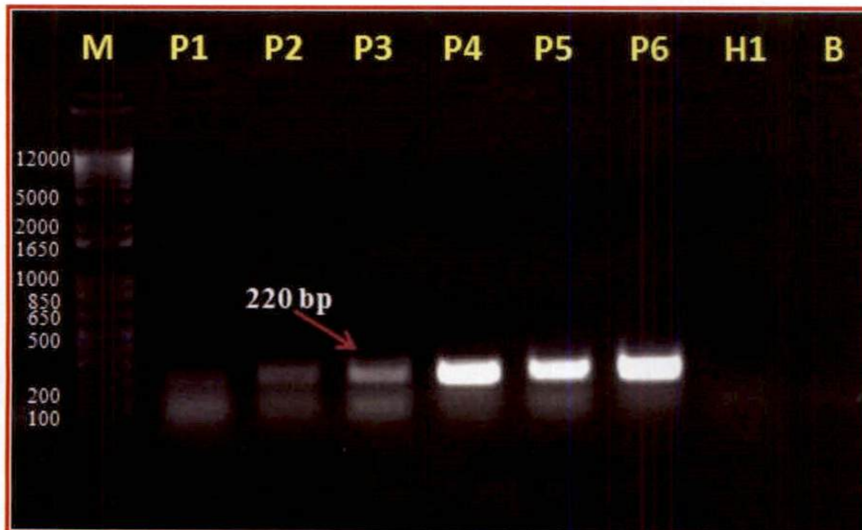

Plate 14: Characterization of *Cardamom mosaic virus* with CP-5

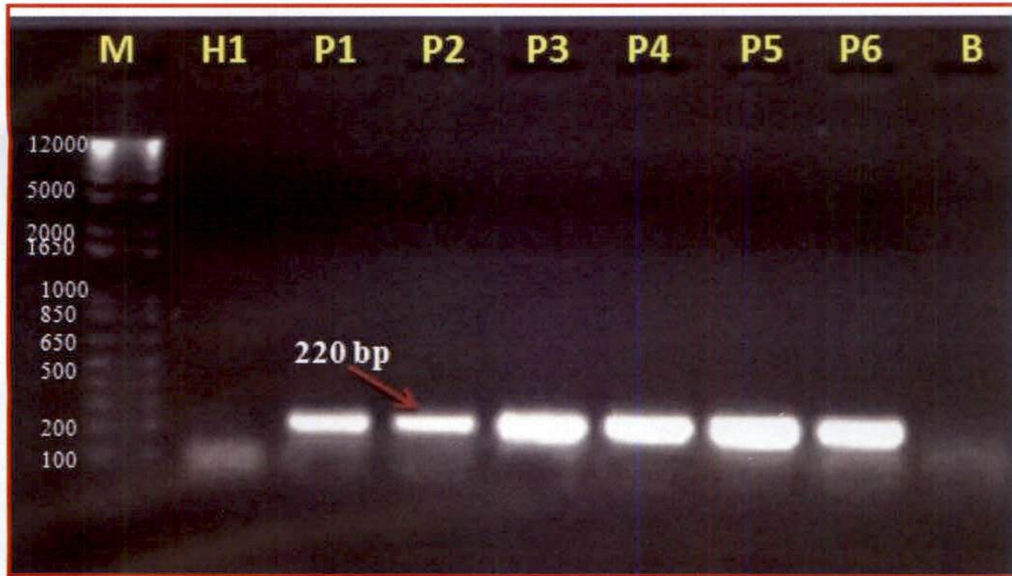

Plate 15: Characterization of *Katte mosaic virus* with CP-7

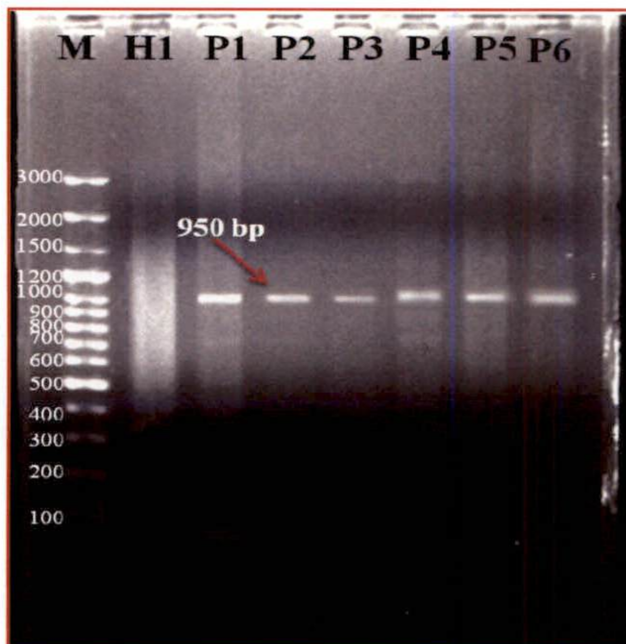

Plate 16: Characterization of *Cardamom mosaic virus* with CP-9

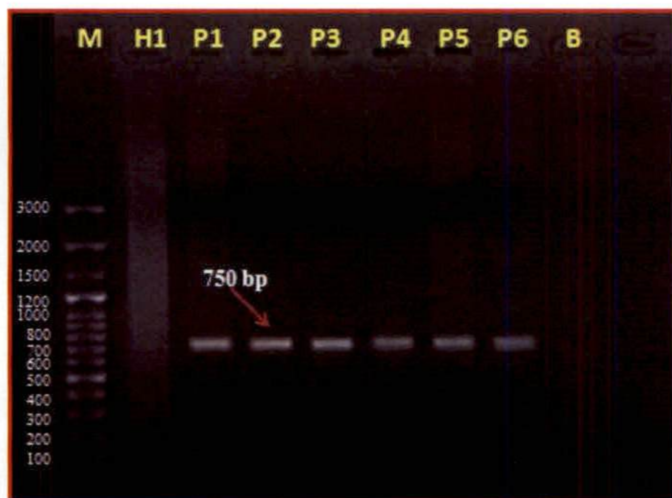

Plate 17: Characterization of *Katte mosaic virus* with CP-10

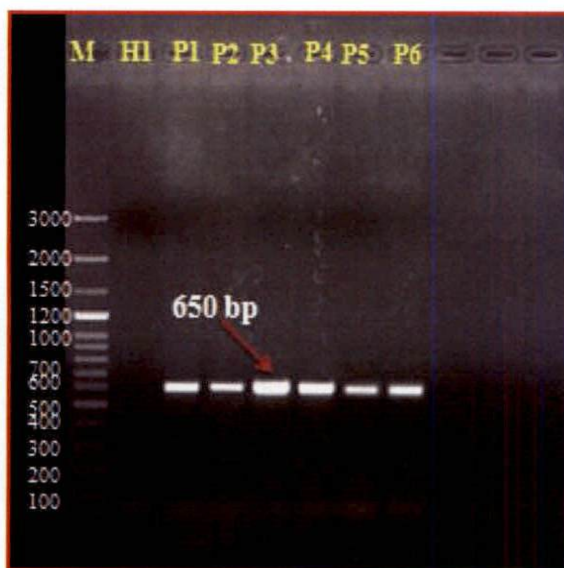

Plate 18: Characterization of *Katte mosaic virus* with CP-11

P1- Ambalavayal (Infected) **P5- Pampadumpara (Infected)**

P2- Meppadi (Infected) **P6- Myladumpara (Infected)**

P3- Paravalam (Infected) **H1- Healthy samples**

P4- Irettyar (Infected) **B- Blank** **M- Ladder (100bp plus)**

successfully used for the characterization of katte infected plants from the healthy plants.

Table 15: Details of amplification pattern of coat protein targeting primers

SL.No.	Primer name	Expected Band size (in base pairs)	Observed band size (in base pairs)
1	CP-1	250	220
2	CP-2	250	220
3	**CP-3**	**250**	**No amplification**
4	CP-4	250	220
5	CP-5	250	220
6	**CP-6**	**250**	**No amplification**
7	CP-7	250	220
8	**CP-8**	**250**	**No amplification**
9	CP-9	800	950
10	CP-10	700	750
11	CP-11	600	650

4.3.6. Elution and reamplification of viral specific amplicon

The viral specific amplicon amplified by CP-2, CP-10 and CP-11 was eluted from the gel and reamplified with the same set of primers as the initial PCR reaction and analyzed in the 1 per cent agarose gel. The agarose gel electrophoresis of eluted product showed the similar banding pattern as in initial PCR amplification (Plate 19-20).

4.3.7. Sequencing of viral amplicon

The reamplified viral specific amplicon of Meppadi and Pampadumpara region amplified by CP-2 and viral specific amplicon of Meppadi region amplified by

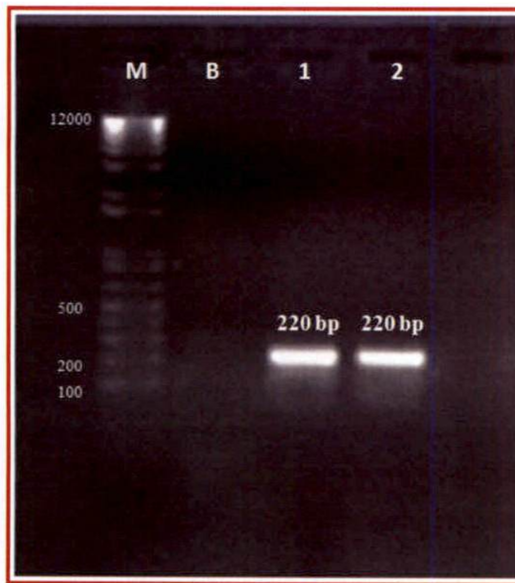

Plate 19: Gel profile of reamplified eluted DNA using CP-2 primer

M- Ladder (1Kb plus)   Lane-1: Blank

Lane-2: Reamplified product of Pampadumpara region

Lane-3: Reamplified product of Meppadi region

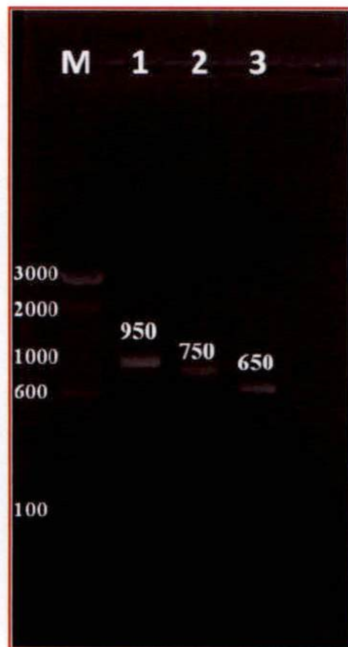

Plate-20: Gel profile of reamplified eluted DNA of different primers

M- Ladder (100 bp plus)

Lane-1: Reamplified product of CP-9 primer (Meppadi)

Lane-2: Reamplified product of CP-10 primer (Pampadumpara)

Lane-3: Reamplified product of CP-11 primer (Myladumpara)

CP-9, Pampadumpara region amplified by CP-10 and Myladumpara region amplified by CP-11 were sequenced (Scigenome, Kochi). The nucleotide sequence obtained after sequencing was 178, 228, 950, 750 and 650 bp respectively. (Figure 7-11).

4.4. *Insilico* analysis

The nucleotide sequence of coat protein obtained after sequencing was further annotated using NCBI- BLAST (n and x) programme to check the sequence homology.

4.4.1. *Insilico* analysis of Sequence-1

The coat protein sequence of Meppadi region amplified by CP-2 primer was submitted to NCBI- BLASTn (Figure 12) and BLASTx (Figure13). The BLASTn results revealed that the nucleotide sequence of Meppadi region has 98 per cent similarity with *Cardamom mosaic virus* (Table 16).Whereas, BLASTx results shows that the sequence has 98 per cent similarity with coat protein of Indian Cardamom mosaic virus (Table 17).

4.4.2. *Insilico* analysis of Sequence-2

The coat protein sequence of Pampadumpara region amplified by CP-2 primer was submitted to NCBI- BLASTn (Figure 14) and BLASTx (Figure 15). The BLASTn results revealed that the nucleotide sequence of has 98 to 100 per cent similarity with capsid protein gene of Cardamom mosaic virus isolates of Meppadi and Pampadumpara region (Table 18).Whereas, BLAST results shows that the sequence has 58 per cent similarity with coat protein of Cardamom mosaic virus (Table 19).

ATGCTTTCAATCCATTTTTCAAATTGCAAAGTTGTGGCAAGCTG
GGATGACTGTGTGAACTGCTCTTTCGCGATGCTATTTATAATTC
TCCTGTTCCAAACTCGTCTACCTTTGATGCGTGGGTTATGGAAA
TGGCCTCGGAAAGAGCCGTTCACACAGTCATCCCAGCTTGA

Figure 7: Coat protein sequence of *Cardamom mosaic virus* from Meppadi region amplified by CP-2 primer (Sequrnce-1)

TGCTTTCAATCCATTTTTCTGCAAAGCCTACACCAAAGATGCTG
AGTTGTGGCAAGCTGGGATGACTGTGTGAACTTTCGCGATGCT
ATTTATCAGGTGGGTTGTTTTCAACAATCCCAATTAATTCTCCT
GTTCCAAACTCGTCTACCTTTGATGCGTGGGTTATGGAAATGG
CCTCGGAAAGAGCCCCACAACTTTGCAATTTGAAAAATGGAAT
CCCAGCTTGA

Figure 8: Coat protein sequence of *Cardamom mosaic virus* from Pampadumpara region amplified by CP-2 primer (Sequence-2)

TGAACAGTTGACAACCAATTTGGTGATTTGCCTCCAGGTGAAGGCGGAGATGGA
GATGATGCAGATATTCAATGGAGAATCCCACCCACACCAAAAACGCTGAACCAT
TTTCACACCCACGCGTCAAAGGTAGACGAATCTGGAATAGAAGGATCATAAATA
GCATTGCAAAGGAGCAATTTACACAATCATCTCAACTCGCCACAACGTTGCAGTT
TGAAAAATGGGCTGAGAGTGTTAGGAAGAGTCTTGGAACACCAAATGAAGGTGA
TTTTCAAATTTATTTGACATCATGGTGCCTCTGGTGTGCAAATAATGGAACATCTT
CAGAAGTCGATGTTAATCAAAATATGGAAATTCACTCAGGTGGTTCGTTTTCAAC
AATCCCAATATCTATTTTGTTGAACCAGCAGTTCAGATGTCGACGTGTCTCTCATA
ACTTGTTTTGCTACGCTGCACCTTTCCATCGAGCAGCATGACACGTTGGTGTCCAG
ATCCAATTGCTGCTGCTTTTCCTTGATTTAATTGTTCGCGAACTGTCTTTGGCATTC
CATTTGTCTGAACGCAAAAATCAAAAGCATAGGGAATCATTGCAAGTTGCGTGTA
GCCTCTTTTGGTTCCCCATGCAGTCATCTTTCCTCCTTTCGCAAGTATTTGTGAAGT
TACATCACTGAGATGCCGCATAATCTTCCTGAGCCCTCCATTCTGAACTGCTGGTT
CAACAAAATATAGATATTGGGATTGTTGAAAACGAACCACCTGAGTGAATTTCCA
TATTTTGATTAACATCGACTTCTGAAGATGTTCCATTATTTGCACACCAGAGGCAC
CATGATGTCAAATAAATTTGAAAATCACCTTCATTTGGTGTTCCAAGACTCTTCCT
AACACTCTCAGCCCATTTTTCAAACTGCAACGTTGTGGCGAGTTGAGATGATTGT
GTAAATTGCTCCTTTGCAATGCTATTTATGATCCTTCTATTCCAGATTCGTCTACCT

Figure 9: Coat protein sequence of *Cardamom mosaic virus* from Meppadi region amplified by CP-9 primer (Sequence-3)

AAATAGCATCGCGAAAGAGCAGTTCACACAGTCATCCCAGCTTGCCACA
ACTTTGCAATTTGAAAAATGGATTGAAAGCATTCGGAAAAGTCTTGGCAC
CCCAAATGAAGGCGACTTCCAGATCTATTTGACATCATGGTGCCTCTGGT
GTGCAAATAATGGGACATCTCAGAAGTTGATGTGAATCAGAACATGGAA
ATTCACTCAGGTGGGTTGTTTTCAACAATCCCAATATCCATTTTCGTGAGC
CGGCAGTTCAGAATGGAGGGCTTAAAAAGATTATGCGCATAGCAGCTGC
CTTCCCTTGATTTAATTGTTCACGAACTGTTTTTGGCATTCCATTTGTTTGA
ACGCAGAAATCAAAAGCATAGGGGATCATTGCAAGCTGTGTGTAACCTC
TTTTGGTCCCCCATGCCGTCATTTTTCCTCCTTTTGCAAGTATCTGTGTGGT
CACATCACTAAGATGCCGCATAATCTTTCTAAGCCCTCCATTCTGAACTG
CCGGCTCAACGAAAATGGATATTGGGATTGTTGAAAACAACCCACCTGA
GTGAATTTCCATGTTCTGATTCACATCAACTTCTGAGGATGTCCCATTATT
TGCACACCAGAGGCACCATGATGTCAAATAGATCTGGAAGTCGCCTTCAT
TTGGGGTGCCAAGACTTTTCCGAATGCTTTCAATCATTTTTCAAATTGCAA
AGTTGTGGCAAGCTGGGATGACTGTGTGAAC

Figure 10: Coat protein sequence of *Cardamom mosaic virus* from Meppadi region amplified by CP-10 primer (Sequence-4)

TGCAAAGGAGCAATTTACACAATCATCTCAACTCGCCACAACGTTGCAGT
TTGAAAAATGGGCTGAGAGTGTTAGGAAGAGTCTTGGAACACCAAATGA
AGGTGATTTTCAAATTTATTTGACATCATGGTGCCTCTGGTGTGCAAATA
ATGGAACATCTTCAGAAGTCGATGTTAATCAAAATATGGAAATTCACTCA
GGTGGTTCGTTTTCAACAATCCCAATATCTATTTTTGTTGAACCAGCAGTT
CATAATGGAGGGCTCAGGAAGATTATGCGGCATCTCAGTGATGTAACTTC
ACAAATACTTGCGAAAGGAGGAAAGATGACTGCATGGGGAACCAAAAG
AGGCTACACGCAACTTGCAATGATTCCCTATGCTTTTGATTTTTGCGTTCA
GACAAATGGAATGCCAAAGACAGTTCGCGAACAATTAAATCAAGGAAAA
GCAGCAGCAATTGGATCTGGACACCAACGTGTCATGCTGCTCGATGGAA
AGGTGCAATCCAATTGCTGCTGCTTTTCCTTGATTTAATTGTTCGCGAACT
GTCTTTGGCATTCCATTTGTCTGAACGCAAAAATCAAAAGCATAGGGAAT
CATTGCAAGTTGCGTGTAGCCTCTTTTGGTTCCCCATGCAGTCATCTTTCC
TCCTTTCGCAAGTATTTGTGAAGT

Figure 11: Coat protein sequence of *Cardamom mosaic virus* from Meppadi region amplified by CP-11 primer (Sequence-5)

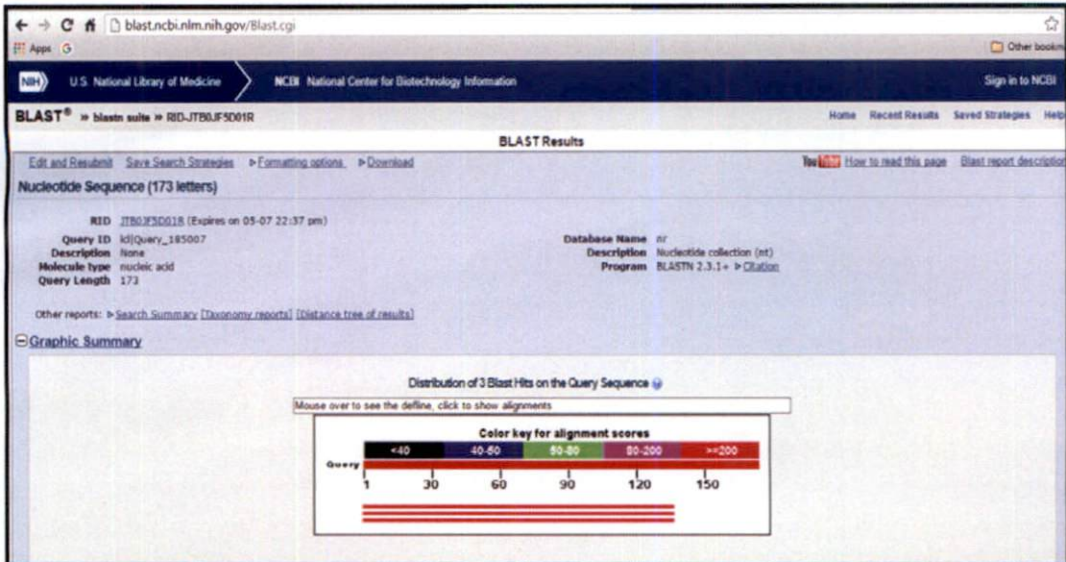

Figure 12: Description page of BLASTn for Sequence-1 (Meppadi region)

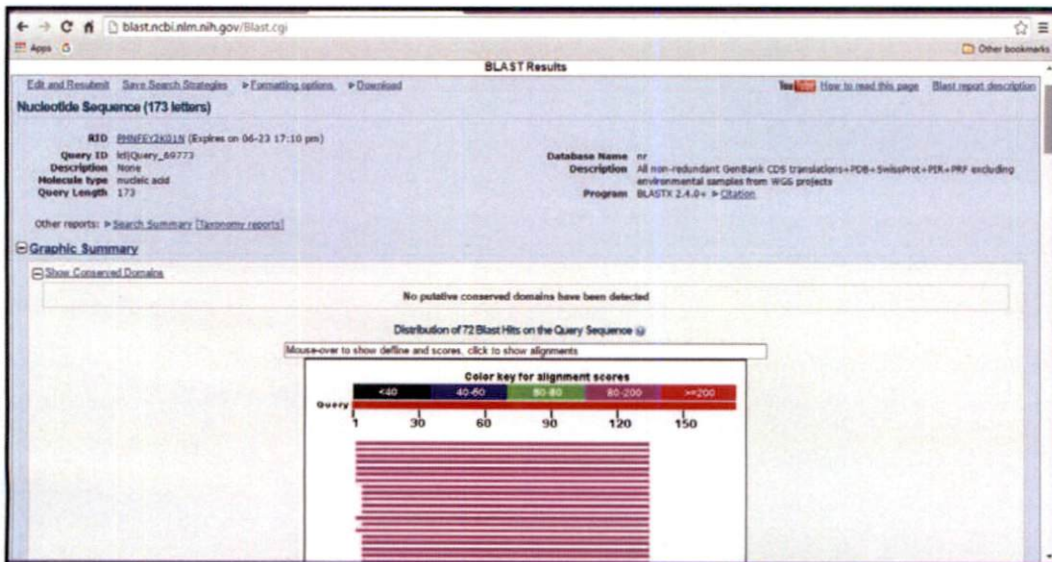

Figure 13: Description page of BLASTx for Sequence-1 (Meppadi region)

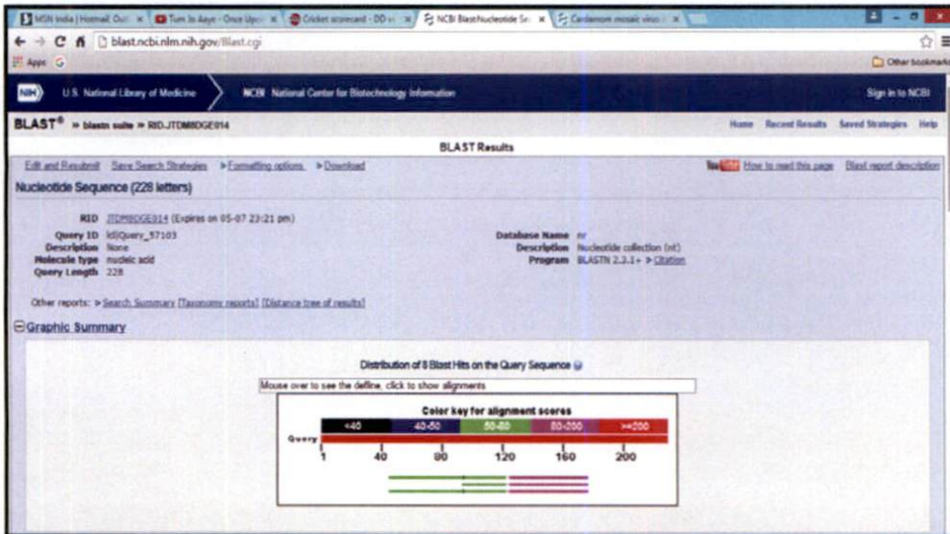

Figure 14: Description page of BLASTn for Sequence-2 (Pampadumpara region)

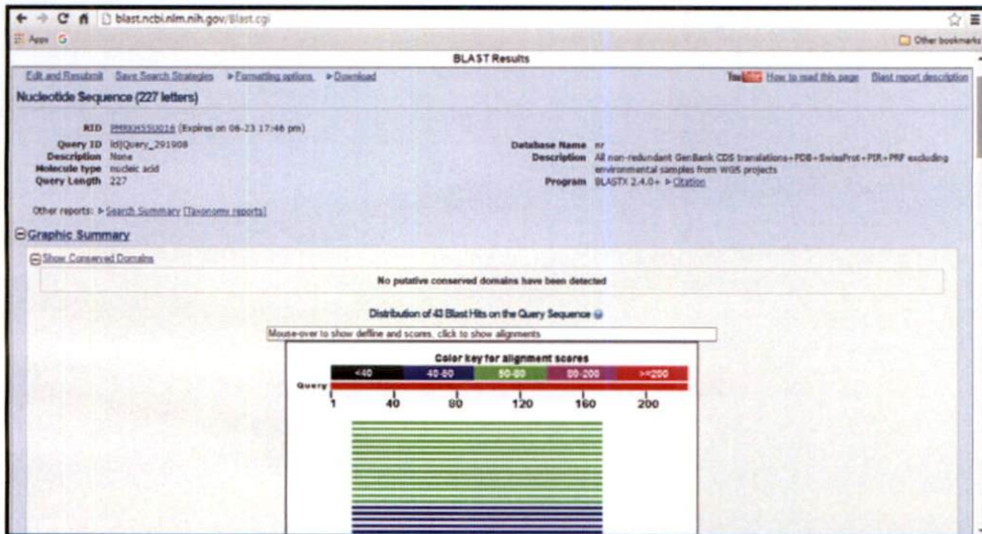

Figure 15: Description page of BLASTx for Sequence-2 (Pampadumpara region)

Table 16: BLASTn analysis of sequence-1 (Meppadi region)

Accession	Description	Max. score	Query coverage (%)	E value	Max. identity
JN544080.1	*Cardamom mosaic virus* isolate Meppadi capsid protein gene, partial cds	235	78%	2e-58	98%
JN544077.1	*Cardamom mosaic virus* isolate Pampadumpara capsid protein gene, partial cds	235	78%	2e-58	98%
AJ308476.1	Indian *Cardamom mosaic virus* partial cp gene for coat protein, genomic RNA, isolate Kursupara	235	78%	2e-58	98%

Table 17: BLASTx analysis of sequence-1 (Meppadi region)

Accession	Description	Max. score	Query coverage (%)	E value	Max. identity
CAC29004.1	Coat protein(Indian *Cardamom mosaic virus*)	94.0	76%	4e-22	98%
AFN85099.1	capsid protein (*Cardamom mosaic virus*)	94.0	76%	4e-22	98%
AFN85102.1	capsid protein [*Cardamom mosaic virus*]	94.0	76%	4e-22	98%

Table 18: BLASTn analysis of sequence-2 (Pampadumpara region)

Accession	Description	Max. score	Query coverage (%)	E value	Max. identity
JN544080.1	*Cardamom mosaic virus* isolate Meppadi capsid protein gene, partial cds	97.1	56	1e-16	100%
JN544077.1	*Cardamom mosaic virus* isolate Pampadumpara capsid protein gene, partial cds	91.6	35	6e-15	98%
AJ308476.1	Indian *Cardamom mosaic virus* partial cp gene for coat protein, genomic RNA, isolate Kursupara	97.1	56	1e-16	100%

Table 19: BLASTx analysis of sequence-2 (Pampadumpara region)

Accession	Description	Max. score	Query coverage (%)	E value	Max. identity
AFN85099.1	capsid protein [*Cardamom mosaic virus*]	55.5	70	2e-07	58%
CAC29004.1	coat protein [Indian *Cardamom mosaic virus*]	55.5	70	2e-07	58%
AFN85102.1	capsid protein [*Cardamom mosaic virus*]	55.5	70	2e-07	58%

4.4.3. *Insilico* analysis of Sequence-3 (Meppadi region)

The coat protein sequence of Meppadi region amplified by CP-9 primer was submitted to NCBI- BLASTn (Figure16) and BLASTx (Figure 17). The BLASTn results revealed that the nucleotide sequence of has 88 to 93 per cent similarity with capsid protein gene of *Cardamom mosaic virus* isolates (Table 20).Whereas, BLAST results shows that the sequence has 98 to 100 per cent similarity with coat protein of *Cardamom mosaic virus* (Table 21) and having 3 conserved domine of potyvirus.

4.4.4. *Insilico* analysis of Sequence-4 (Pampadumpara region)

The coat protein sequence of Pampadumpara region amplified by CP-9 primer was submitted to NCBI- BLASTn (Figure 18) and BLASTx (Figure 19). The BLASTn results revealed that the nucleotide sequence of has 91 to 98 per cent similarity with capsid protein gene of Cardamom mosaic virus isolates of Pampadumpara and Meppadi (Table 22).Whereas, BLASTx results shows that the sequence has 92 to 98 per cent similarity with coat protein of *Cardamom mosaic virus* (Table 23) and having 2 conserved domine of potyvirus.

4.4.5. *Insilico* analysis of Sequence-5 (Pampadumpara region)

The coat protein sequence of Meppadi region amplified by CP-9 primer was submitted to NCBI- BLASTn (Figure 20) and BLASTx (Figure 21). The BLASTn results revealed that the nucleotide sequence of has 88 to 92 per cent similarity with capsid protein gene of Cardamom mosaic virus isolates of Pampadumpara and Meppadi (Table 24).Whereas, BLAST results shows that the sequence has 90 to 95 per cent similarity with coat protein of *Cardamom mosaic virus* (Table 25) and having 1 conserved domine of potyvirus.

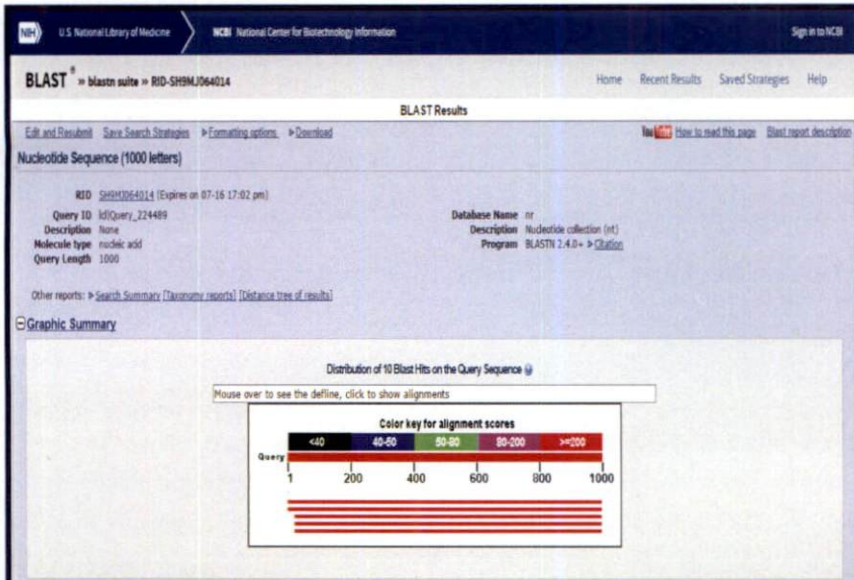

Figure 16: Description page of BLASTn for Sequence-3 (Meppadi region)

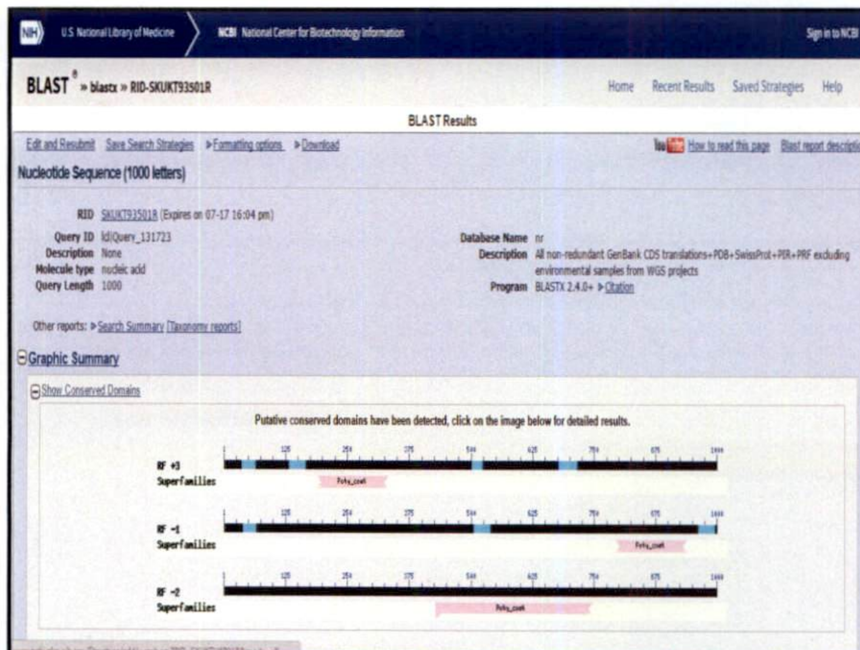

Figure 17: Description page of BLASTx for Sequence-3 (Meppadi region)

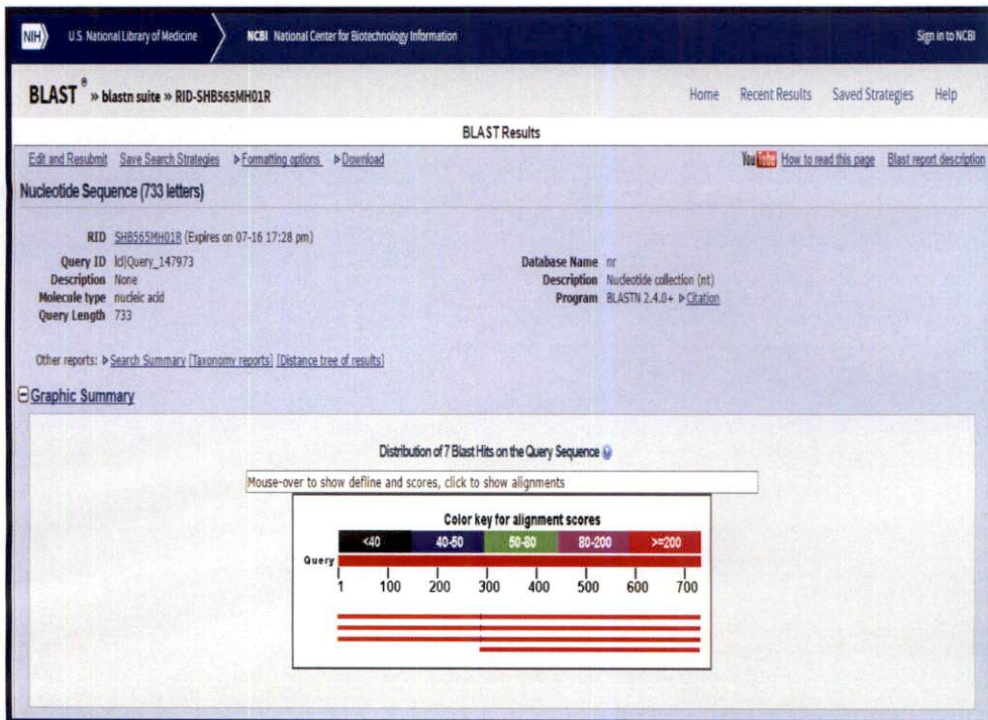

Figure 18: Description page of BLASTn for Sequence-4 (Pampadumpara region)

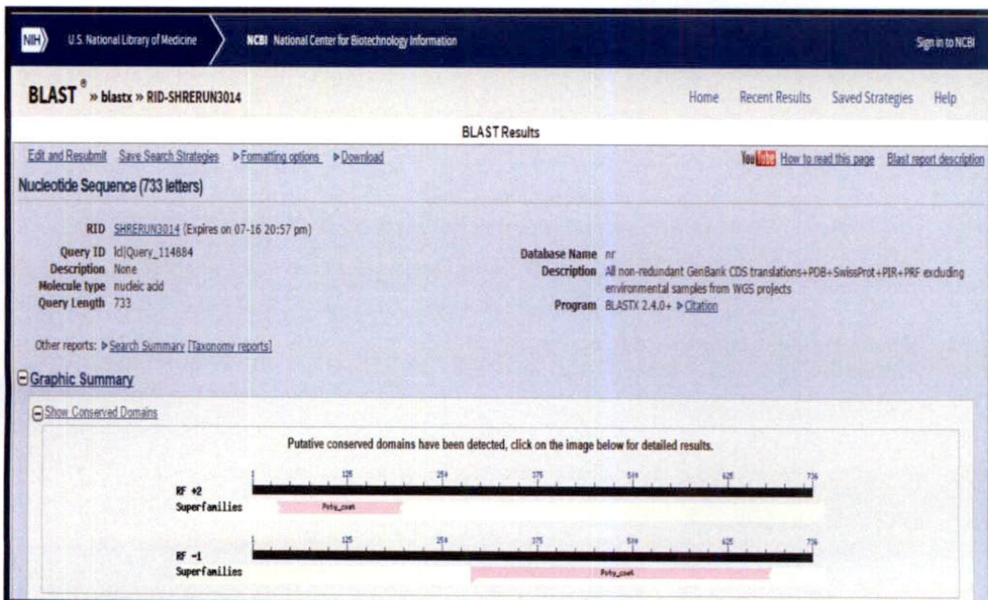

Figure 19: Description page of BLASTx for Sequence-4 (Pampadumpara region)

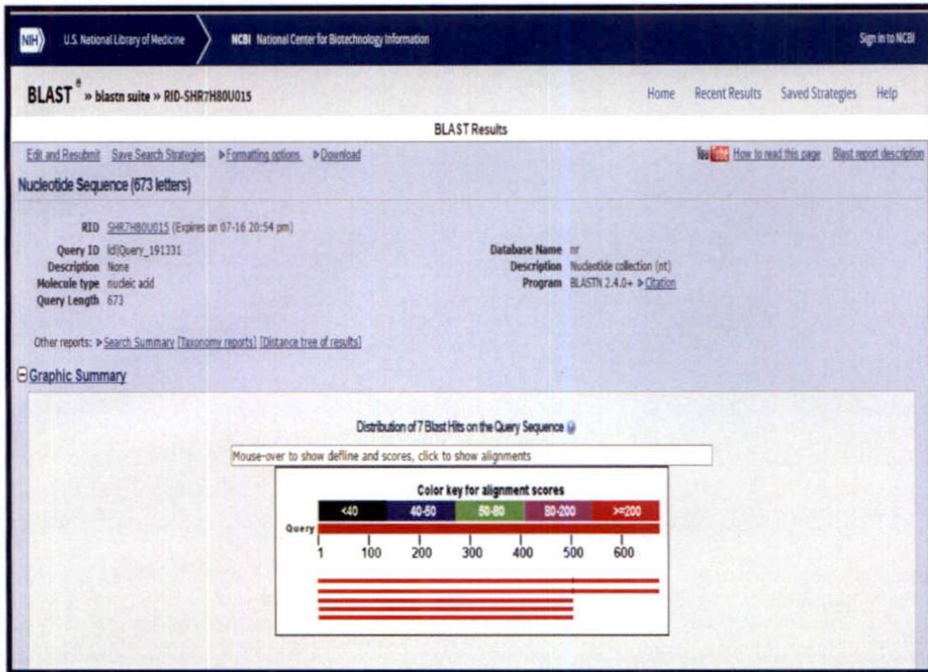

Figure 20: Description page of BLASTn for Sequence-5 (Myladumpara region)

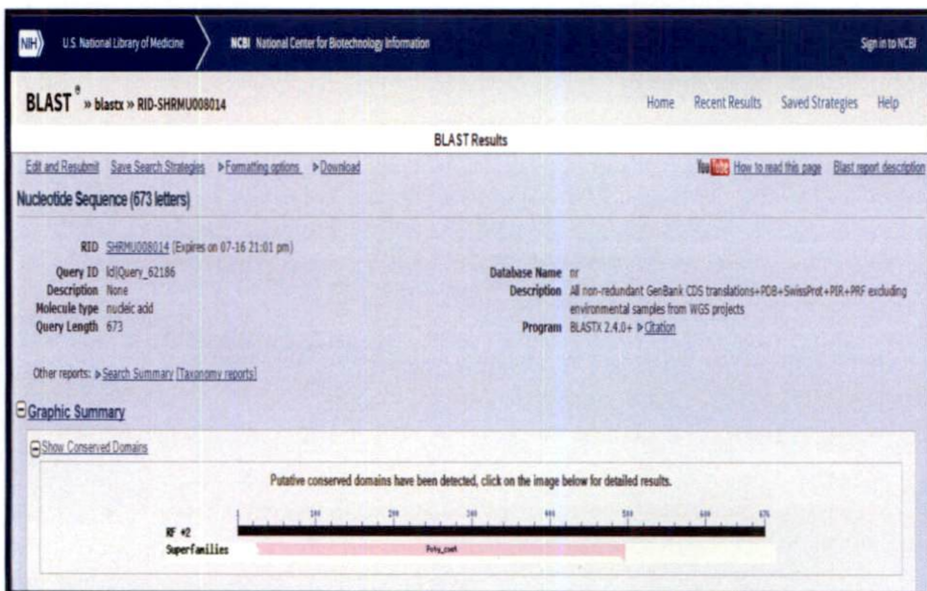

Figure 21: Description page of BLASTx for Sequence-5 (Myladumpara region)

Table 20: BLASTn analysis of sequence-3 (Meppadi region)

Accession	Description	Max. score	Query coverage (%)	E value	Max. identity
AJ312774.1	Indian *Cardamom mosaic virus* partial cp gene for coat protein, genomic RNA	848	99	0.0	93%
AJ308477.1	Indian *Cardamom mosaic virus* partial cp gene for coat protein, genomic RNA, isolate Vandiperiyar	732	99	0.0	89%
JN544080.1	*Cardamom mosaic virus* isolate Meppadi capsid protein gene, partial cds	699	97	0.0	88%
JN544077.1	*Cardamom mosaic virus* isolate Pampadumpara capsid protein gene, partial cds	688	97	0.0	88%

Table 21: BLASTx analysis of sequence-3 (Meppadi region)

Accession	Description	Max. score	Query coverage (%)	E value	Max. identity
CAC37402.1	Coat protein [Indian *Cardamom mosaic virus*]	214	93	4e-95	100%
CAC29005.1	Coat protein [Indian cardamom mosaic virus]	210	95	5e-94	98%
AFN85099.1	Capsid protein [*Cardamom mosaic virus*]	213	86	5e-94	98%
CAC29004.1	Coat protein [Indian *Cardamom mosaic virus*]	213	86	5e-94	98%

Table 22: BLASTn analysis of sequence-4 (Pampadumpara region)

Accession	**Description**	**Max. score**	**Query coverage (%)**	**E value**	**Max. identity**
AJ308476.1	*Indian Cardamom mosaic virus* partial cp gene for coat protein, genomic RNA, isolate Kursupara	785	100	0.0	98%
JN544077.1	*Cardamom mosaic virus* isolate Pampadumpara capsid protein gene, partial cds	780	100	0.0	98%
JN544080.1	*Cardamom mosaic virus* isolate Meppadi capsid protein gene, partial cds	765	100	0.0	98%
JN544078.1	*Cardamom mosaic virus* isolate Thadiyankudisai capsid protein gene, partial cds	590	60	2e-164	91%

Table 23: BLASTx analysis of sequence-4 (Pampadumpara region)

Accession	**Description**	**Max. score**	**Query coverage (%)**	**E value**	**Max. identity**
AFN85099.1	capsid protein [*Cardamom mosaic virus*]	281	93	3e-92	98%
CAC29004.1	coat protein [Indian *Cardamom mosaic virus*]	281	93	3e-92	98%
AFN85100.1	capsid protein [*Cardamom mosaic virus*]	281	93	3e-92	98%
AFN85102.1	capsid protein [*Cardamom mosaic virus*]	281	93	3e-92	98%

Table 24: BLASTn analysis of sequence-5 (Myladumpara region)

Accession	Description	Max. score	Query coverage (%)	E value	Max. identity
AJ312774.1	Indian *Cardamom mosaic virus* partial cp gene for coat protein, genomic RNA	713	99	0.0	92%
AJ308477.1	Indian *Cardamom mosaic virus* partial cp gene for coat protein, genomic RNA, isolate Vandiperiyar	647	99	0.0	90%
JN544080.1	*Cardamom mosaic virus* isolate Meppadi capsid protein gene, partial cds	608	74	5e-170	88%
JN544077.1	*Cardamom mosaic virus* isolate Pampadumpara capsid protein gene, partial cds	603	74	2e-168	88%

Table 25: BLASTx analysis of sequence-5 (Myladumpara region)

Accession	Description	Max. score	Query coverage (%)	E value	Max. identity
CAC37402.1	coat protein [Indian *Cardamom mosaic virus*]	348	99	6e-119	95%
CAC29005.1	coat protein [Indian *Cardamom mosaic virus*]	345	99	4e-118	98%
AFN85100.1	coat protein [Indian *Cardamom mosaic virus*]	344	99	1e-117	94%
CAC29004.1	coat protein [Indian *Cardamom mosaic virus*]	344	99	1e-117	94%

4.5. Phylogenetic analysis

All the six isolates of *Katte mosaic virus* collected from different locations of Kerala were amplified with CP-9 primer and amplicons were sequenced which was further used for phylogenetic analysis. The phylogenetic tree was developed using MEGA.7 software by utilizing neibhourhood joining method. The result of phylogenetic analysis revealed that the isolates of Ambalavayal, Meppadi and Myladumpara are closely related and related to isolates of Irettyar and Paravalam whereas, Pampadumpara isolate showed more variation to the above isolates (Fig.-22).

Myladumpara
Ambalavayal
Meppadi
Irettyar
Paravalam
Pampadumpara

Figure 22: Phylogenetic analysis of coat protein nucleotide sequence of *Cardamom mosaic virus* isolates collected from different location of Kerala.

Discussion

5. DISCUSSION

Cardamom (*Elettaria cardamomum* Melton), the queen of spices is one of the most important spice crop cultivated in hill tracks of Kerala, Karnataka and Tamil nadu in India. It is mostly cultivated vegetatively through rhizomes which make them susceptible to many pest and diseases mostly through viral vectors. Among the viral diseases, Katte disease of cardamom caused by *Katte mosaic virus* or *Cardamom mosaic virus* (CdMV) is one of the most destructive disease of cardamom causing almost 100 per cent yield loss in cardamom plantation throughout the world. In India, the katte disease of cardamom was first reported from South India by Buchana in 1907 and later Mayne in 1951 confirmed. The disease is widely distributed in cardamom growing tracks of South India. The disease caused by *Katte mosaic virus* occurs at any stage of crop development but if infection occurs at early stage of crop growth results in 100 per cent yield loss. The disease is primarily transmitted through infected planting materials such as rhizome or clumps and secondary through insect vector *Pentalonia nigronervosa* Coq. The disease causes heavy reduction in yield in the cardamom growing tracts of India.

The incidence of disease is sometimes symptomless or shows mild symptoms which often misleads to nutrient deficiency disorders, so identification and diagnosis of virus becomes difficult. Therefore, it is important to develop a quick and accurate diagnostic technique for detection of *Cardamom mosaic virus* to prevent the spread of disease through infected planting material. Hence, present investigation was conducted to develop serological and PCR based methods for identification and characterization of *Katte mosaic virus* of cardamom for easy and early detection. The results of the present investigation are discussed in this chapter.

5.1 Collection and maintenance of infected plant samples

Healthy and infected cardamom plants of variety Njallani green gold were collected from Wayanad and Idukki district of Kerala showing symptoms such as mosaic with chlorotic fleck on the young leaf running parallel from midrib to margin (Uppal *et al.*1945; Rao and Naidu 1973; Dimitman 1984 and Venugopal 1995) and mosaic symptoms on the pseudostem were collected and maintained.

5.2. Purification of *Cardamom mosaic virus* from infected leaves

The purification of CdMV was performed in 0.5 M KP buffer as explained in chapter 3.2.2.1 for antibody production with slight modification. Where, PVP and β-mercaptoethanol was added to the extraction buffer to inhibit the polyphenol oxidase. The protein reacts with oxidized phenolic compounds which lead to reduction in the viral protein content. The earlier reports also suggested that oxidized phenolic compounds interfere with viral protein content in the case of *Solanum nodiflorum mottle sobemo virus* (Chu and Franki, 1983) and *Barley yellow mosaic virus* (Ehlers and Paul, 1986)

The most common problem encountered during virus isolation was viral particle aggregation for which, 0.5 M urea was added in the resuspension buffer.

5.3. SDS-PAGE analysis of partially purified viral protein

SDS-PAGE was performed to confirm the presence of viral coat protein by its molecular weight in the partially purified protein fraction. CBB (Coomassie Brilliant Blue) staining failed for visualization of the band in the gel as the protein concentration in the purified fraction was low. Hence, silver staining was performed which, was more effective over CBB staining. In the present study, staining with silver nitrate revealed the presence of viral coat protein of 37 kDa. The similar results were also reported by Gonsalves *et al.* (1986) and Jacob and Usha (2001) who found three bands in SDS-PAGE analysis with 37.5, 32, 27 kDa

and one band of 38 kDa size respectively which is slightly higher than the present finding. No such bands were observed in the healthy plant samples.

5.4. Development of polyclonal antibody against *Cardamom mosaic virus*

Polyclonal antibody was produced with partially purified coat protein (antigen) against CdMV in 6-9 month old New Zealand white rabbit as mentioned in earlier chapter 3.2.2.3a as mentioned by (Gonsalves *et al.* 1986; Abida, 1995; Eoi *et al.*, 2010; Subhashini, 2013).

Polyclonal antibody was then purified from the blood sample collected from the immunized rabbit as described in the chapter 3.2.2.3b. The purified antiserum was stored in 4^0C and further utilized for ODD assay and indirect ELISA as reported by Eoi *et al.*, (2010) against *Cucumber mosaic virus.*

5.5. Determination of antibody titre

In order to determine the antibody titre, ODD assay was performed. The different dilutions of polyclonal antibody raised against CdMV was used in the ODD assay and the result revealed the 1:10, 1:100 and 1:150 dilution of antibody was able to form antigen antibody complex with coat protein of virus (Antigen). The antibody titre indentified in the present investigation is much higher as the concentration of antigen used for immunization was less in the purified protein samples.

Abida (1995) reported the ODD assay for determining the antibody titre for the antibody produced against plant hormone abscisic acid.

5.6. Detection of *Cardamom mosaic virus* through Indirect ELISA

The result generated conclusively reveals that the partially purified total protein isolated from diseased samples were positive against the antibody developed with 1:10, 1:100 and 1:150 dilution with constant (1:200) dilution of

antirabbit secondary antibody conjugated with APS or HRP. Findings in this study are similar with Gonsalves *et al.* (1986) who detected the CdMV by indirect ELISA using 1:800 and 1:1000 dilution of primary and secondary antibody.

5.7. Serological relationships of *Cardamom mosaic virus* with other viruses

The result generated conclusively revealed that serological relationship of viruses can be used for classifying the poty viruses. The polyclonal antibody of different viruses from different host plant showed significant cross reactivity in indirect ELISA. The presence of antibody to the epitope TRC (trypsin resistant core) region of antigen, which is a conserved amino acid region found in the most of the potyviruses (Hammond, 1989). Moreover, the antibodies produced against the TRC epitope will dissociate the TRC region, which reacts with most of the poty- viruses due to the production of antibody for the conserved region (Shukla *et al.*, 1994).

Findings of indirect ELISA shows the *Cardamom mosaic virus* is serologically related to the *Banana bract mosaic virus* which is a member of the potyvirus but shows no relationship with *Cucumber mosaic virus* and *Banana bunchy top virus*. Smitha (2004) reported that the *Cardamom mosaic virus* has some relationship with *Banana bract mosaic virus* which is similar to the findings in the study conducted.

Gonsalves *et al.* (1986) also reported that *Cardamom mosaic virus* shows serological relationship with *Zucchini yellow mosaic virus*, *Papaya ringspot virus*, *Bean mosaic virus* belongs to family potyviridae which are confirmation to the findings concluding that *Katte mosaic virus* also belongs to potyvirus group.

5.8. Detection of *Cardamom mosaic virus* using RT-PCR

5.8.1. Isolation of total RNA

In this study attempts were made to standardize the protocol for isolation of total RNA from the infected and healthy sample using TRIzol reagent (Sigma-Aldrich) in a single step. Trizol is a mixture of phenol and guanidine isothiocyanate which maintains the integrity of RNA during the process of cell disruption and lyses. In addition the chloroform was added followed by centrifugation leads to separate aqueous phase which contains RNA .Then RNA was precipitated by using isopropanol. The similar method of RNA isolation was followed by Biju *et al.* (2010) and Siljo *et al.*(2013)

This is the most efficient method of isolating the total RNA from the leaf over phenol chloroform extraction method (Simms *et al.*, 1993). The RNA obtained using TRIzol method is of good quality with the OD $_{260/280}$ ranges from 1.9-2.0 when analyzed under nanodrop spectrophotometer.

The overall quality of total RNA isolated from the infected and healthy leaf samples was assessed by electrophoresis, in 2 per cent formaldehyde denaturing gel. The denaturing system used because under normal condition RNA can form different types of secondary structure that prevent its mobility in the gel so separation under normal agarose gel is difficult. In denaturing gel formaldehyde is used to maintain the denatured condition of RNA and finally the bands were visualized using ethidium bromide. This is the most common method of analyzing the quality of total RNA but disadvantage of this method is the use of formaldehyde which is a carcinogenic chemical which requires the proper handling (Mansour *et al.*, 2013). The RNA analyzed in the denaturing gel was having 3 intact (28S, 5S and t-RNA) bands which confirm the purity of RNA without any DNA or protein contamination. Babaurao (2012) and Jacob *et al.* (2003) reported that the quality and quantity of total isolated

from the leaf samples can be assessed by formaldehyde denaturing gel electrophoresis.

5.8.2. cDNA synthesis using RT-PCR

Total RNA isolated from the infected and healthy samples were converted into cDNA (first strand) using Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit. The M-MuLV Reverse Transcriptase (RT) supplied within the kit is required for converting the RNA into cDNA by using Oligodt primer. Most of the RNA transcript has polyA tail at 3' end, oligodt primer binds to polyA tail of RNA transcript and M-MuLV RT act as polymerase and synthesizes DNA using RNA template. The RNase was inhibited using the Ribolock RNase inhibitor to protect RNA template from degradation. The synthesis cDNA was confirmed by synthesizing the second strand using actin primer, a house keeping gene present in most of the eukaryotes. The RNA isolated from infected and healthy samples were converted into cDNA which were further utilized in PCR for characterizing the CdMV with coat protein specific primers. The similar method for the cDNA synthesis was reported by Biju *et al.,* (2010) and Jebasingh (2011) and Baburao (2012).

5.8.3. Primer designing for amplification of coat protein gene and its validation

The primer targeting the coat protein region of CdMV were designed using Primer 3 software. The primer3 software is online and user friendly software which is most commonly used for designing the primer pairs for various application. The Primer3 software consider many factors such as GC content, Tm, product size, length of the primer while selecting the primer pairs (Rozen and Skaletsky, 2000). The published nucleotide sequence of coat protein of CdMV was utilized for designing the primer pairs using primer3 software. Total 11 primers pairs were designed using primer3 software. Biju *et al.* (2010) designed the primers pairs manually by using multiple sequence alignment of CdMV available in NCBI database. Similarly Siljo *et*

al. (2013) was also designed the degenerated primer targeting the coat protein region of the virus.

The primers developed by primer3 software were validated using the software IDT oligoAnalyzer 3.1 software. OligoAnalyzer3.1 software is a calculator which provides useful predictions about the oligonucleotide sequence and their properties under various experimental conditions. The software also helps in selecting the oligonucleotide which can exhibit best performance in biological condition. There seven different parameters which can be analyzed using this software for getting good primers. The physical properties such as GC content, length of oligonucleotide, complementary sequences, oligonucleotide length, content of G and C bases, Tm (melting temperature), extinction coefficient and molecular weight were determined by clicking ANALYZE bottom whereas other parameters such heterodimer, self-dimer (homodimer) and Tm mismatch were determined (Owczarzy *et al.,* 2008). All the 11 primer pairs were validated using this software and further utilized for the amplification of coat protein region of CdMV. The eleven primers designed in the present study using primer 3 software and its validation was first report. Among the designed primers, eight primers were successfully amplifying the coat protein gene in infected samples alone and among them the primer CP-9 generated band size of 950 bp.

5.8.4. Amplification of coat protein region of *Cardamom mosaic virus* using gene specific primer

The second strand of cDNA of infected and healthy samples was amplified with the coat protein specific primer designed in the present study. All the 11 primer pairs were used to amplify the coat protein region of the CdMV. The amplified product of CP-1, 2, 4, 5 and 7 was 220 bp where as the amplified product of CP-9 was 950 bp, CP-10 was 750 bp and CP-11 was 650 bp. These were observed in the infected samples but absent in the healthy samples. Primers such as CP-3, 5 and 6

were unable to amplify the coat protein region of the virus. The product size of 250 bp is very less compared with 750bp band reported by Biju *et al.* (2010) but the product size of CP-10 was similar with the report. Jacob *et al.* (2003) also reported the 1000 bp band with the primers CPn2 and Poty1 + primer which contain the coat protein and 3'UTR region which is similar with the amplified product of CP-9 primer. The genome size of the virus was about 1.8 kb containing 3' untranslated region, Coat protein region and NIB region of the virus (Jacob *et al.*, 2003)

These primers can be successfully used for characterizing the katte infected plant from the healthy samples and can be used for the detection of *Cardamom mosaic virus* and among the eight primers CP-9 was found to best for the identification and characterization of the virus.

5.9. *In-silico* analysis

The virus amplicons of size 220bp, 950bp, 750bp and 650bp generated with primer pairs CP-2, 9, 10 and 11 were eluted from the gel and sequenced. The sequence obtained after sequencing were annotated with database subjected to similarity search using bioinformatics tools such as BLASTn and BLASTx. The BLASTn result showed 80-100 per cent similarity with *Cardamom mosaic virus* where as the result of BLASTx revealed that the sequence has 55-100 per cent similarity in the coat protein of cardamom mosaic virus. The similar findings were reported by Siljo *et al.* (2013) and characterized the Kerala and Tamil nadu isolates in to group 3 with 87.1 to 98.9 per cent similarity.

The phylogenetic analysis was performed using MEGA software. The result revealed that the isolates of Pampadumpara are variable among the isolates collected from the two districts of Kerala. Jacob *et al.* (2003) reported that variation in the coat protein and 3'untranslated region of *Cardamom mosaic virus.* In present study the variation observed may be due to the exchange of planting material from neighboring states.

Overall the methods developed in the present study will be useful in indexing the virus in the tissue cultured plants and in planting material for supply of disease free planting materials to the farmers. The methods can also be used for indentifying the resistant lines or cultivars for large scale production and may be incorporated in the breeding programme. The method will also useful in quarantine management and germplasm management to prevent the spread of the virus in cardamom plantations. In future line, the methodology adopted and developed is time consuming even though it is reliable and sensitive, hence technique such as nano based biosensors could be developed.

Summary

6. SUMMARY

The study entitled "Molecular characterization of *Katte mosaic virus* of cardamom (*Elettaria cardamom* Maton)" was carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Thrissur during the academic year of 2014-2016. The objective of the study was to develop serological and PCR based methods for the identification and characterization of *Katte mosaic virus* of cardamom (*Elettaria cardamom* Maton). The salient finding of the present investigation has been summarized under following sub-heading.

6.1. Collection of planting material

The symptomatic and asymptomatic plants were collected from different locations of different location of Wayanad district of north Kerala and Idukki district of south Kerala. The infected samples were collected based on the symptoms described by Uppal *et al.* (1945) and Capoor (1967) as mosaic pattern with chlorotic streak on the leaves and on the pseudostem. The symptomatic and asymptomatic plants were planted and maintained as per the Package of practices of Kerala agricultural university.

6.2. Detection of *Cardamom mosaic virus* by ELISA

The viral protein was isolated from the six different samples collected from different districts of Kerala using the modified protocol of Gonsalves which was found to be efficient in purifying the virus. The SDS-PAGE analysis of total purified protein from infected and healthy plants resulted in the generation of 37 kDa protein band which was present alone in infected plants.

The purified protein from the infected plants was used as antigen for the production of production of polyclonal antibody against CdMV in rabbit. The blood samples from the immunized rabbit were collected and antibody was purified. The ODD assay was performed to determine the titre of the antibody and result of the

ODD assay shows that 1:10, 1:100 and 1:150 dilution of primary antibody were formed antigen-antibody complex.

The indirect ELISA was carried out with 1:10, 1:100 and 1:150 dilution of primary antibody and 1:200 dilution of secondary antibody and found that the virus can easily detected in the total protein extract of infected leaves with 1:100 dilution of primary antibody. The indirect ELISA was also used for investigating the serological relationship of the CdMV with other viruses. The result of indirect ELISA revealed that the total protein of infected cardamom leaves cross reacted with the antibody of BBrMV which shows that CdMV was serologically related to BBrMV.

6.2. Detection of *Cardamom mosaic virus* by RT-PCR

The total RNA was isolated from the infected and healthy plant using Trizol and converted into cDNA using cDNA synthesis kit. Total 11 numbers of primers were designed for the amplification of coat protein gene of the virus using primer3 software and validated using IDT Oligo analyzer 3.1. The developed primers were used in the PCR for synthesis of second strand of cDNA and for characterization. Out of 11 primers, 8 primers were able to amplify the coat protein gene of the virus in infected plants. The PCR product of 250 bp, 650 bp, 750 bp and 1000 bp were observed in infected samples but not in healthy plant samples.

6.3. *Insilico* analysis

The viral amplicon of 250, 650, 750, 1000 base pairs were eluted, reamplified and sequenced. The nucleotide sequence was then annotated with data base using BLASTn and BLASTx. The result of BLASTn analysis showed that the nucleotide sequences had 80 to 100 per cent similarity with CdMV whereas the result of BLASTx analysis revealed 55-100 per cent similarity to the coat protein of CdMV. The phylogenetic analysis was also performed using MEGA.7 software and phylogenetic tree was drawn using nucleotide sequences. The result of phylogenetic

analysis revealed that the isolates of Pampadumpara are variable among the six isolates collected from different locations of Kerala.

The methods developed in the present study are useful in detecting the *Katte mosaic virus* or *Cardamom mosaic virus* in the infected leaf samples of cardamom. The method will be useful in virus indexing, quarantine management in germplasm exchange, germplasm management, supply of disease free planting materials to the farmers and also for selecting the resistant line or cultivar for large scale production or incorporation in breeding for crop improvement.

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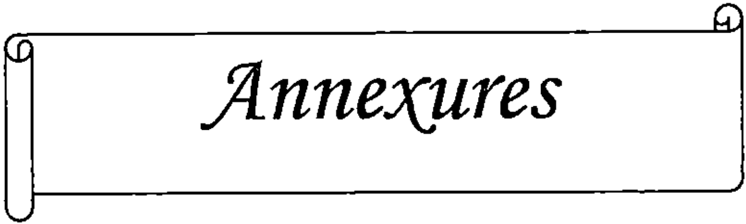
Annexures

ANNEXURE I

List of laboratory equipment used for the studies

SDS-PAGE unit	:	Biored
Electrophoresis unit	:	Biored
Thermocycleer	:	Agilent
Laminar air flow	:	Labline industries
Shaker incubator	:	JEIO Tceh, Korea
ELISA reader	:	VERSA max, USA
High speed refrigerated centrifuge	:	Kubota
Geldoc XR	:	Biored

ANNEXURE II

Chemical composition of buffers used for virus purification

1. Extraction buffer

0.5 M KP buffer (pH-7.1)	
Potassium dihydrogen phosphate	68.0 g
Dipotassium orthophosphate	87.07 g
0.01 M EDTA	3.72 g
0.1% β-Mercaptoethanol	1.0 ml
2% PVP	20.0 g
Distilled water	Upto 1000 ml

2. Resuspension buffer

0.01 M KP buffer	
5 mM EDTA	0.372 g
0.5 M urea	30.03 g
Distilled water	Upto 1000 ml

ANNEXURE III

Chemical composition of buffer and staining solution used for SDS-PAGE

1) Acrlamide stock solution

Acrylamide	29.2 g
N,N-methyl bisacrylamide	0.8 g
Distilled water	Upto 100 ml

2) SDS (10 %)

SDS	10 g
Distilled water	Upto 100 ml

3) Ammonium persulphate (APS) -10%

APS	0.1 g
Distilled water	Upto 1 ml

4) Protein loading dye (1X)

Tris-HCl	50mM
β-Mercaptoethanol	0.1%
SDS	10%
Bromophenol blue	0.1%
Glycerol	10%

5) Electrode buffer or Tank buffer (5x) pH-8.3

Tris-base	15 g
Glycine	72.0 g
SDS	5.0 g
Distilled water	Upto 1000 ml

6) Resolving gel Buffer (1.5 M Tris-HCl, pH-8.8)

Tris-base	18.15
Distilled water	Upto 100 ml

7) Stacking gel buffer (1 M Tris-HCl, pH-6.8)

Tris-base	12
Distilled water	Upto 100 ml

8) Fixer solution and Stop solution

Ethanol	150 ml
Acetic acid	7.5 ml
Distilled water	Upto 1500 ml

9) Staining solution

Silver nitrate	2.25 g
Formaldehyde	2.25 ml
Distilled water	Upto 1500 ml

10) Developer solution

Sodium hydroxide	22.5 g
Formaldehyde	2.25 ml
Distilled water	Upto 1500 ml

ANNEXURE IV

Chemical composition of buffer used for indirect ELISA

1. Coating buffer (PBS - pH 7.4)

Sodium chloride	8.0 g
Potassium dihydrogen phosphate	0.2 g
Disodium hydrogen phosphate	1.1 g
Potassium chloride	0.2 g
Water	1000 ml

2. Washing buffer(PBS-T)

PBS buffer (pH- 7.4)	1000 ml
Tween 20 (0.05%)	0.5 ml

3. Substrate solution (pH 9.8)

Diethanolamine	97 ml
Sodium azide	0.2 g

Water 800 ml Add HCI to give pH 9.8

4. Blocking solution

PBS-T	100 ml
BSA (0.2%)	0.2 gm

5. Antibody dilution buffer

PBS-T	100ml
BSA (0.2%)	0.2gm
PVP (2%)	2gm

ANNEXURE V

Chemical composition of buffer and dyes used for gel electrophoresis

1) Loading/tracking dye

Bromophenol blue	0.25%
Xylene cyanol	0.25%
Glycerol	30%

The dye was prepared and kept in fridge at 4 ^{0}C

2) Ethidium bromide (intercalating dye)

The dye was prepared as a stock solution of 10 mg/ml in water and was stored at room temperature in an amber coloured bottle.

3) TAE buffer (pH-8.0) – 50X

Tris-base	242.0 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH-8.0)	100 ml
Distilled water	1000 ml

The solution was prepared and stored at room temperature

4) MOPS buffer (pH-7) – 10X

200 mM MOPS buffer	41.85 g
80 mM Sodium acetate	6.56 g
10 mM EDTA	3.725
DEPC water	1000 ml

The solution was prepared in DEPC treated water and stored at room temperature.

Abstract