Development of recombinant coat protein for immunodetection of *Cucumber mosaic virus* infecting banana

By

Alan C. Antony

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THESIS

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DECLARATION

I, hereby declare that the thesis entitled **"Development of recombinant coat protein for immunodetection of *Cucumber mosaic virus* infecting banana"** is a bonafide record of research done by me during the course of research and that it has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

Alan C. Antony

2017-11-037

Vellanikkara

Date: 20-08-2019

CERTIFICATE

Certified that this thesis entitled **"Development of recombinant coat protein for immunodetection of *Cucumber mosaic virus* infecting banana"** is a record of research work done independently by **Mr. Alan C. Antony (2017-11-037)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

Vellanikkara
Date: 17.08.19

Dr. Vimi Louis
(Chairperson, Advisory Committee)
Professor (Plant Pathology)
Banana Research Station, Kannara
KAU, Thrissur

CERTIFICATE

We, the undersigned members of the advisory committee of **Mr. Alan C. Antony (2017-11-037)**, a candidate for the degree of **Master of Science in Agriculture**, with major field in **Plant Pathology**, agree that the thesis entitled **"Development of recombinant coat protein for immunodetection of *Cucumber mosaic virus* infecting banana"** may be submitted by Mr. Alan C. Antony, in partial fulfillment of the requirement for the degree.

Dr.Vimi Louis
(Chairman, Advisory Committee)
Professor (Plant Pathology)
Banana Research Station, Kannara
KAU, Thrissur

Dr. Anita Cherian K.
(Member, Advisory Committee)
Professor and Head
Dept. of Plant Pathology
College of Hortriculture, Vellanikkara

Dr. M. Mini
(Member, Advisory Committee)
Professor and Head
Dept. of Veterinary Microbiology
College of Veterinary and Animal
Sciences, Mannuthy

Dr. Babu, T.D
(Member, Advisory Committee)
Associate Professor
Dept. of Biochemistry,
Amala Cancer Research Centre,

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Alan C Antony

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Introduction

1. INTRODUCTION

Banana (*Musa* spp.) is one of the most important fruit crops in India, especially in southern regions. The plant plays an amicable role in religious and cultural occasions, hence the crop has named as 'Kalpatharu' (Plant of virtues). Viruses infecting banana can have a direct effect on production by reducing plant growth and yield. It causes yield losses up to 40-100 per cent (Gambley and Thomas, 2001). Economically important viruses infecting banana are *Banana bunchy top virus*, *Banana streak virus*, *Banana bract mosaic virus* and *Cucumber mosaic virus* (CMV).

Banana viruses can also have important indirect effects by restricting germplasm movement and predisposing plants to damage by other biotic and abiotic stress factors. The viral diseases differ from those caused by fungal and bacterial pathogens in two fundamental and critical respects. The first and the most important, is that viral infections cannot be eliminated by chemical treatment (chemotherapy), which means that control of viral diseases must be based on preventing infection rather than on curing the disease. The second difference is that, whereas fungal and bacterial diseases may cause dramatic short-term damage, viral infections often result in long-term devastating effects since the clonal progeny of infected plants are automatically infected by the virus.

As virus diseases cannot be controlled using chemicals, detection at the early stages is the only amicable method for its control. The use of virus free planting material is the most important measure for avoiding virus diseases of banana. Cultivation of tissue culture banana is very popular among banana growers in Kerala. Hence, virus indexing is an ineviTable practice to ensure quality of tissue culture plants.

Infectious chlorosis caused by *Cucumber mosaic virus* is cosmopolitan, and is found in all banana growing areas. This is an emerging viral disease in Kerala, which causes leaf distortion, stunting of plant and yield reduction.

Cucumber mosaic virus taxonomically grouped under family *Bromovirdae*, which contains five genera *i.e.*, *Alfamovirus*, *Ilavirus*, *Cucumovirus*, *Oleavirus* and *Bromovirus*. Several members of *Bromoviridae* are economically important as far as farmers fields are concerned. Most of the Cucumoviruses have narrow host range, but *Cucumber mosaic virus* is an exception. Among members of *Bromoviridae*, *Cucumovirus* and *Bromovirus* possess efficient mechanism of transmission through aphids. *Aphis craccivora* and *A. gossypii*, are the species which can efficiently transmit the virus in a non-persistent manner. In some Bromoviruses, beetles can be acted as vectors.

Techniques for effective management and control of *Cucumber mosaic virus* differ from other viruses infecting banana, because these viruses differ in their biological properties, epidemiology and distribution. It is essential to know the characteristic of each virus for effective control of viral diseases and for development of reliable virus detection methods. Biological, serological and molecular methods like, Direct antigen coating enzyme linked immunosorbent assay (DAC- ELISA) and Reverse transcriptase polymerase chain reaction (RT-PCR) are used to detect CMV from field.

Antiserum production is an essential pre- requisite for serological detection. In earlier periods, this was done using purified virus but it is usually a cumbersome procedure in terms of purity and concentration of the final preparation. Contamination of antigens with plant proteins or other viral proteins in the case of mixed infection and presence of inhibitory compounds are also the drawbacks of this method. Coat protein (CP) region of CMV in banana is sufficient enough to provide a reliable method for the detection of virus. Thus, recombinant coat protein based antiserum is an efficient remedy for the above mentioned dilemma.

Developing antiserum against local virus isolates will be useful for enhancing the efficacy of indexing and ultimately eliminating the virus from the tissue culture plants.

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Hence, the present investigation was undertaken to produce and purify the coat protein gene of *Cucumber mosaic virus* in *Escherichia coli* (*E. coli*) with the project entitled 'Development of recombinant coat protein for immunodetection of *Cucumber mosaic virus* infecting banana'.

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Review of literature

2. REVIEW OF LITERATURE

Cucumber mosaic virus is an emerging plant virus having, wide host range and distribution in temperate and tropical areas. It was first reported simultaneously by Doolittle (1916) and Jagger (1916) in cucumber.

This chapter comprises the chronicle of genomic, post-genomic and diagnostic studies of *Cucumber mosaic virus* and the same has reviewed under various headlines.

2.1. DISTRIBUTION

Cucumber mosaic virus is geographically wide spread and having broad host range, including some annual crops in temperate zones, tropical regions and Mediterranean countries (Tomlinson, 1987). The symptoms of infectious chlorosis in banana *Musa* spp. AAA Cavendish group cv. Williams was collected from northern tropical Kimberley region of Western Australia. *Cucumber mosaic virus* (CMV) was first reported in detail on cucumber and other cucurbits, but is now known to occur worldwide in both temperate and tropical climates, affecting many agricultural and horticultural crops (Zitter and Murphy, 2009). *Cucumber mosaic virus* has been reported in Australia, North America, New Zealand, Europe, Africa and Iran (Roosinck *et al*., 1999; Sokhandan-Bashir *et al*., 2012).

Cucumber mosaic virus isolates were phylogenetically analysed and the subgroup I has subdivided into IA and IB. Among the subgroups, subgroup 1B of *Cucumber mosaic virus* is limited to Asia, and the other two subgroups (*i.e*., 1A and 2) are distributed worldwide (Sivaprasad *et al*., 2016).

In India, CMV occurrence has been reported in commercially grown flowers and spices such as chrysanthemum (Srivastava *et al*., 1992); carnation (Raj *et al*., 1993); black pepper (Sharma *et al*., 2001); and periwinkle (*Catharanthus roseus*) (Samad *et al*., 2008). Bhadramurthy (2008) reported that

CMV causes mosaic symptoms in vanilla. The virus has been reported in *Oxalis corymbosa* in Aligarh, India (Sheikh *et al.*, 2013).

According to Estelitta *et al.* (1996) and Mujtaba (2017), CMV is an emerging threat in Kerala, especially where cucurbitaceous vegeTables are cultivated as intercrops in banana orchards; in banana it causes bunch weight reduction, varied between 45- 62 per cent. In Kerala, infection of CMV was noticed in banana varieties such as Karpooravally, Nendran, Palayankodan, Peykunnan, Kosthabontha, Mottapoovan, Bhimkhel, Dhakhinsagar, Madhuraga, Rasthali and *Musa ornate* (KAU, 2016).

2.2. SYMPTOMATOLOGY

Severe epinasty, downward bending of the petiole and leaf surface along with leaf reduction, is common in early infection of cucurbits. Plants infected early in the season are severely stunted, leaves are malformed, and fruit are unmarkeTable because of pronounced rugosity (roughness) on the fruit surface (Agrios, 2005). Infection of vining crops, such as muskmelon, shows severely stunted growing tips, and although fruit may not show symptoms. Tomatoes infected with CMV are often stunted and bushy with short internodes and malformed leaves known as fern leaf. Distinctive symptom of CMV infection in tomato is shoestring of leaf blades. The upper leaves can show symptoms while those in the mid-section of the plant appear normal.

Srivastava *et al.* (1992) recorded the symptoms caused by CMV in chrysanthemum. Various types of symptoms recorded were purplish tinge on leaflets, mid rib yellowing, spiny margins, yellow mosaic with green veins, yellow and necrotic spots, severe mosaic and vein yellowing. Madhubala *et al.* (2005) reported the first incidence of CMV in vanilla (*Vanilla planifolia*), which causes various symptoms *viz.*, mosaic, leaf distortion and stunting. Bhadramurthy *et al.* (2009) reported that CMV could cause mosaic, leaf distortion and stunting on paprika (*Capsicum annuum* L.). In pumpkin, CMV infected at early stage

expressed severe foliar and fruit mosaic pattern which made the crop unmarkeTable (Zitikaite *et al.*, 2011).

2.2.1. Symptoms of *Cucumber mosaic virus* infection in Banana

The first signs of *Cucumber mosaic virus* infection in banana were noticed in NSW, Australia (Magee, 1930). The disease was named as infectious chlorosis, heart rot, virus sheath rot, cucumber mosaic and banana mosaic, according to the external symptoms caused by virus (Stover, 1972).

Mosaic patterns or discontinuous linear streaking bands, extending from leaf margin to midrib are the characteristic symptoms of infectious chlorosis. The presence of dead or dying suckers is noticed in advanced cases referred as heart rot resulting from rotting of heart leaf and central portion of pseudostem. Primarily, infected banana plants develop severe mosaic symptoms in young plants showing broadly streaked chlorotic or yellowish green bands and patches or chlorotic mottling distributed in patches over the leaf lamina (Niblett *et al.*, 1994). Banana mosaic or infectious chlorosis is one of the important and widely distributed viral diseases of banana. Banana mosaic is categorised as cosmopolitan and is found wherever bananas are grown. Even at low titre of virus, the whole leaf may become chlorotic due to decreased chlorophyll production and breakdown of chloroplasts (Dheepa and Paranjothi, 2010)

Stover (1972) stated that, symptoms and strains of *Cucumber mosaic virus* are positively correlated. The symptoms occurred sporadically and majority of the leaves appeared healthy. The virus was capable enough to cause infection in internal tissues of pseudostem. The plants might die most likely, if it is infected with severe strains immediately after planting. Rodoni *et al.* (1997) conducted a survey at Coimbatore and Tiruchchirappalli regions of southern India for *Banana bract mosaic virus* and witnessed banana cultivars like, Pisang Awak, Poovan, and Matti hybrids with symptoms similar to those caused by CMV, including diamond shaped streaks, mosaic on the leaf lamina and mottled pattern on the

petioles of leaves. In severely infected leaves, stunted appearance and distortion of leaf blades were noticed.

Banana plants infected with CMV cause mosaic or chlorosis, yellow stripes and ring spots in the leaf lamina. Deformation and curling of leaves, rosette appearance of leaf arrangement and obvious inter-veinal chlorosis are also symptoms of the disease (Tripathy, 2016). The expression of symptoms can be influenced by virus strain and temperature. If the temperature is below 24°C severe symptoms are observed. Necrosis of emerging cigar leaves leads to varying degree of necrosis in leaf lamina (Sivaprasad *et al.,* 2016).

In Kerala, the disease is considered as an emerging one and the symptoms were documented. Infected leaves were narrower and smaller than normal; the infected plants were dwarf and lag behind in growth. The plants masked the symptoms of infection and act as a virus reservoir. Infected leaves produce parallel chlorotic streaks on younger leaves; later leaves become distorted, irregular wavy leaf margin along with necrotic tissues (KAU, 2016). Rolling of leaf margins, twisting and bunching of leaves at the crown and a rigid erectness in newly emerged leaves are noticed in Kerala (Mujtaba, 2017).

2.3. GENOME ORGANISATION

Bromoviridae members are spherical or quasi-spherical, having T=3 icosahedral symmetry, and a diameter of 26–35 nm (genera *viz.*, *Anulavirus*, *Bromovirus, Cucumovirus* and *Ilarvirus*) or bacilliform (genera *viz., Alfamovirus*, *Ilarvirus* and *Oleavirus*) having constant diameters of 18–26 nm and lengths from 30 to 85 nm, within a species. Total genome length is approximately 8 kb. Genomes consist of three linear, positive sense ssRNAs with 5'-terminal cap. The 3'termini are not polyadenylated, but generally are highly conserved within a species or isolate. They are either tRNA-like and can be amino acylated (genera *Bromovirus* and *Cucumovirus*) or form other structures that are not amino acylated (genera *Alfamovirus, Anulavirus, Ilarvirus* and *Oleavirus*). Major viral proteins associated with the *Bromoviridae* family are enlisted in Table 2.1.

Table 2.1: Details of viral protein encoded in *Bromoviridae*

Protein	Size (kDa)	mRNA	Function
1a	102.7–125.8	RNA 1	Methyltransferase (Mtr), helicase
2a	78.9–96.7	RNA 2	Replicase protein with a polymerase domain (RdRp)
3a	30.5–36.5	RNA 3	Cell to cell movement
Coat protein or Capsid protein	19.8–26.2	subgenomic RNA(Sub-genomic RNA-4)	Encapsidation, intercellular movement

Cucumber mosaic virus is the type species of the genus *Cucumovirus* in the family *Bromoviridae*. It contains three spherical particles, each approximately 28 nm in diameter. A third nonstructural protein P2b is expressed, as subgenomic RNA (sgRNA), from RNA2 and functions in cell-to-cell movement and post-transcriptional gene silencing and RNA 3 represents nonstructural movement protein (P3a, cell-to-cell MP) and the structural capsid protein or coat protein (P3b, CP) that is expressed via subgenomic RNA (*i.e.*, RNA4) (Hull, 2009).

Ribonucleic acid molecules are enclosed within a protective protein coat with each being a distinct single spherical-shaped particle. Thus, mature CMV consists of three spherical particles, one particle containing RNA 1, another containing RNA 2 and the third containing RNA 3. The RNA 3 particle contains a fourth RNA strand, known as RNA 4 (~1,030 nucleotides) otherwise called sub genomic strand, which encodes the coat protein gene and from which the CMV coat protein is produced (Zitter and Murphy, 2009). The genomic organisation of CMV is depicted in figure 2.1.

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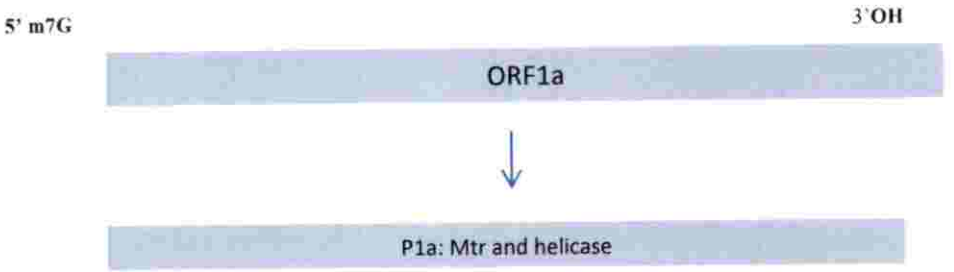
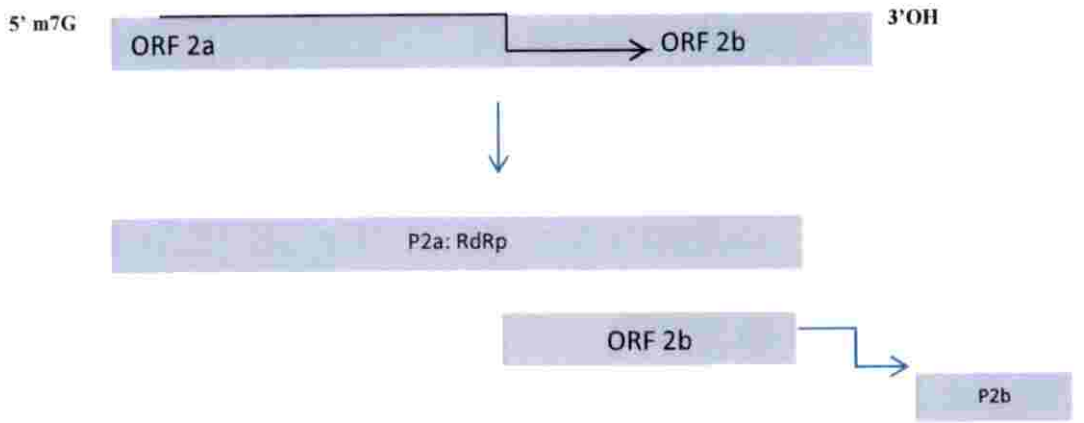
RNA 1 (3357 bp)



RNA 2 (3050 bp)



RNA 3 (2216 bp)

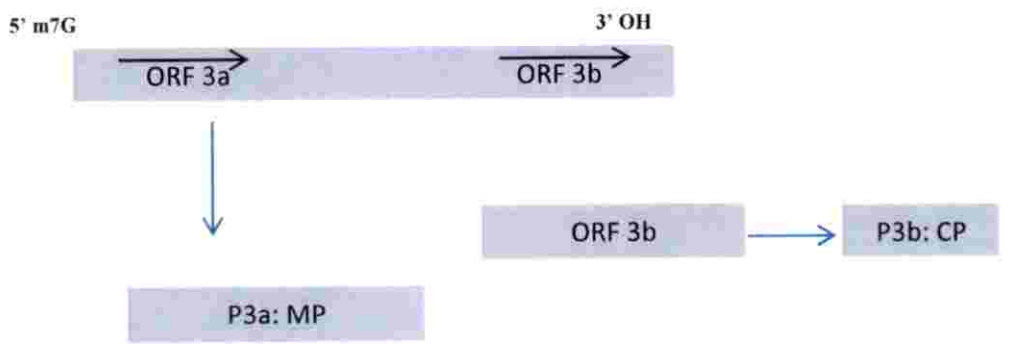


Fig 2.1: Genomic organisation of *Cucumber mosaic virus*

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2.6. DETECTION OF THE VIRUS

The main techniques applied for the diagnosis of plant viruses include biological assays, electron microscopy, serological tests, viral double-stranded RNA analysis, nucleic acid hybridization, RT-PCR and other molecular means. When large numbers of samples have to be tested, double antibody sandwich-enzyme linked immunosorbent assay is widely used. Virus detection methods have upgraded greatly in recent years with the development of more versatile and accurate serological and molecular diagnostic techniques such as real-time polymerase chain reaction, which can be applied directly in the field or microarrays.

Nucleic acid probes , RT-PCR and ELISA have been used for detection and differentiation of CMV isolates along with biological characterisation, serology and electron microscopy in chrysanthemum (Srivastava *et al.*, 1992), carnation (Raj *et al.*, 1993), banana (Kiranmai *et al.*, 1996), geranium (Verma *et al.*, 2004), gladiolus, pepper and vanilla (Madhubala *et al.*, 2005), and anthurium (Miura *et al.*, 2013).

2.6.1. Molecular diagnosis

Polymerase chain reaction is the fundamental techniques to molecular diagnosis of plant viral diseases. It is widely used by researchers in scientific field such as molecular cloning, gene manipulation, gene expression analysis, sequencing, and mutagenesis (Lundberg *et al.*, 1991; Makkouk and Kumari, 2006; Verkuil *et al.*, 2008). Polymerase chain reeaction has popularised as a diagnostic tool to detect diseases. It is developed as a technique to identify DNA viruses, whereas RT- PCR is used to detect RNA viruses which it include reverse transcription of RNA followed by normal PCR (Ghangal *et al.*, 2009; Jeong *et al.*, 2014).

Molecular techniques are powerful and popular methods used for detection of plant viruses and viroids. Reverse transcriptase polymerase chain is a rapid and

sensitive detection tool for most plant viruses including members of the genus Cucumovirus (Choi *et al*, 1999). The RT-PCR is the most sensitive assay among all, in comparision with ELISA and dot blot tests (Hu *et al*., 1995). Reverse transcriptase PCR method was established to detect seed-borne infection and seed transmission frequency of CMV in pepper seed. The absence of non-specific band in healthy, seeds indicates RT-PCR can be used for detecting CMV infection in pepper seeds (Ali and Kobayashi, 2010). The RT-PCR in turn is used for genomic and post genomic studies of viruses. Amplification of the CP gene resulted at ~657 bp fragment in various crops infected with CMV (Khan *et al*., 2012; El-Borollosy and Hassan, 2014).

Sudhakar *et al*. (2006), detected virus infecting tomato by RT-PCR and restriction fragment length polymorphism analysis (RFLP). Zein and Miyatake in 2009 amplified coat protein region of virus infecting chrysanthemum cultivars (~ 650 bp) using CMV specific primers in RT-PCR. Khan *et al*. (2012) reported the association of CMV with mosaic disease of banana by RT-PCR with a CP band size of 657 bp. Ali *et al*. (2012) performed RT- PCR using CMV coat protein gene specific primers. Amplification was obtained at ~650bp. Shetti *et al*. (2014) obtained an amplicon of CMV- CP at 657 bp from infected cucumber plant samples. Molecular detection has standardised for detection of CMV infecting *Oxalis corymbosa* (Sheikh *et al*., 2013). Southern hybridisation test is is used for sensitive detection of CMV from gladiolus leaf and corms (Pandey, 2015).

2.6.1.1. Molecular detection of virus variability

Deoxy ribonucleic acid sequencing is the process of determining the nucleic acid sequence. It refers as method or technology that is used to determine the order of the four nitrogen bases *viz*., adenine, guanine, cytosine, and thymine.

The sequence of the CP gene of the CMV from paprika (*Capsicum annuum* L.) contained a single open reading frame of 657 nucleotides potentially coding for 218 amino acids (Bhadramurthy *et al*., 2009).

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Indian isolates of CMV, infecting various crops were sequenced and found out its homology with subgroup II of CMV (Kumar *et al.*, 2005; Kumari *et al.*, 2013). High sequence identities and evolutionary tie in coat protein gene has been observed with CMV isolate from Kerala (Mujtaba, 2017).

2.6.2. Serological diagnosis

Detection of plant viruses based on symptoms are of limited value in certain condition. So, identification of the viruses by serological methods will be of more accurate, reliable, less time consuming and also cost effective (Dheepa and Paranjothi, 2010)

Enzyme Linked Immuno-Sorbent Assay has been successfully used for the large scale detection of plant viral diseases (Clark and Adams, 1977). It is relatively inexpensive, sensitive, rapid, simple, reliable and suitable for the testing of many samples simultaneously (Clark, 1981). Several serological methods are available for the detection of plant viruses. Serological methods should meet the requirements like, safety, specificity, speed, high sensitivity, reproducibility, and affordability. Enzyme Linked Immuno Sorbent Assay was used to detect *Grapevine Leafroll Associated Closterovirus* infection (Ling *et al.*, 2000). Additionally, more specific antibodies are a pre-requisite for the application of the extremely sensitive Immunocapture-Reverse Transcriptase-Polymerase Chain reaction (IC- RT- PCR) technique (Komorowska and Malinowski, 2009). Another emerging serological diagnostic method is the lateral flow test, eg. immunostrip test based on the dot immunobinding assay (DIBA), a useful alternative to microtitre plate ELISA. The DIBA has about the same sensitivity as ELISA but has additional advantages of simplicity and quick completion in large numbers of samples (Zein and Miyatake, 2009). Though it is relatively expensive, it is a rapid means of screening crops for the presence of viruses.

Different serological assays used for the diagnosis of CMV are immunodiffusion (Scott, 1968), tube and ring precipitin tests (Mink *et al.*, 1975), western blotting (Towbin *et al.*, 1979), SDS immunodiffusion in agarose gel

(Purcifull *et al.*, 1981). Kiranmai *et al.* (1996) have demonstrated potential applicability of DAC-ELISA in largescale indexing of banana for CMV infection. *Cucumber mosaic virus* isolates are detected using Triple Antibody Sandwitch Enzyme Linked Immune-Sorbent Assay (TAS-ELISA) and IC-RT-PCR (Yu *et al.*, 2005). Pandey (2015) standardised DIBA and ELISA with expressed CMV-coat protein and CMV infected plants using the antibodies raised against the virus, with optimum titre values at 1: 100 dilution of antigen and 1: 1000 dilution of primary and secondary antibody.

Espino *et al.* (1989) and Geering and Thomas (1996) reported the use of ELISA tests for the accurate detection of *Banana bunchy top virus* and *Banana bract mosaic virus* with monoclonal antibodies.

In tomato plants, CMV exhibited severe mosaic, leaf puckering and stunted growth and the virus was detected by ELISA (Sudhakar *et al.*, 2006). Aglave *et al.* (2007) standardised DAC-ELISA for the detection of CMV in Ardhapuri variety of banana. The virus was immunogenic and antiserum with titre of 1:6000 was raised and used in DAS-ELISA. Direct antigen coating-ELISA was standardised with antiserum dilution of 1:4000; with alkaline phsophatase enzyme labelled IgG (1:10,000) and antigen dilution of 1:10 and 1:100 was optimum for the detection of CMV in leaf, sucker and plants. Khan *et al.* (2012) confirmed CMV infection in cucumber by IC-RT- PCR.

Wu and Su (1990) developed plate-trapped antigen (PTA) -ELISA using monoclonal antibodies, to detect BBTV. Agindotan *et al.* (2003) reported the use of high-titred monoclonal antibodies for *Banana streak virus* (BSV), which could detect all the isolates of BSV. The IC-PCR is more sensitive than immune-electron microscopy (IEM) for detecting typical BSV, while IEM showed similar sensitivity as TAS-ELISA by sap dilution end-point analyses (Agindotan *et al.*2003). *Cucumber mosaic virus* causing mosaic, leaf distortion and stunting on paprika (*Capsicum annuum* L.) was detected in India by DAC-ELISA

(Bhadramurthy *et al.*, 2009). Detection of BSV has been problematic due to serological and genomic heterogeneity of virus isolates (Selvarajan *et al.*, 2016).

Hosseinzadeh *et al.* (2012) detected CMV by DAS- ELISA in 10 crops *viz.*, tomato, pea, watermelon, tobacco, broad bean, soybean, squash, eggplant, cucumber and lettuce. Among these, the highest and the lowest CMV infection was associated with watermelon (62.44 per cent) and lettuce (Zero per cent), respectively. Shetti *et al.*, (2014) standardised direct plate ELISA and DIBA for detection of CMV at 1:20 and 1:50 dilutions of crude and ultra-purified antigen respectively, at 1:1000 dilution of both primary and secondary antibody.

Detection of CMV has been done using antisera developed against recombinant coat protein (rCP) of the virus (Khan *et al.*, 2012; EI- Borollosy and Hassan, 2014).

2.7. PROTEIN EXPRESSION AND PURIFICATION

E. coli BL21 (DE3) pLysS is the most commonly used expression host, which is a derivative of *E. coli* BL21(DE3). DE3 is an arrangement of T7 RNA Polymerase gene, under the control of LacUV 5 promotor on a phage genome and pLysS is a plasmid that encodes T7 lysozyme gene. The T7 RNA lysozyme bind to T7 RNA polymerase gene, and block the induction until the addition of IPTG. After the addition of IPTG, number of T7 RNA polymerase gene increases and overcomes the inhibition of LysS (Rosano and Ceccarelli, 2014).

2.7.1. Protein expression and purification for scientific purposes

Yusibov *et al.* (1996) cloned the coat protein of *Alfalfa mosaic virus* (AMV) and expressed in *E. coli*/ pTrcHisB system. About half of the expressed recombinant coat protein (rCP) was soluble upon extraction and the other half was insoluble. Western blot analysis confirmed the identity of the rCP and protoplast infectivity assays indicated that the rCP was biologically active in an early event of AMV infection, called genome activation. Jacob and Usha (2002) expressed *Cardamom mosaic virus* (CdMV) coat protein in *Escherichia coli* and observed

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its assembly into filamentous aggregates. CdMV- CP gene was amplified using gene specific primers and the amplicon was cloned to pProEX HTb/ *E. coli* system. The expression was confirmed by SDS- PAGE and western blotting using CdMV specific antibodies.

Hochuli *et al.* (1987); Chow (2006) and Hartley (2006) has standardized the protocols for cloning of virus coat protein gene in expression vectors and purification of recombinant protein.

Rostami *et al.* (2014) observed the formation of virus like particles (VLPs) of *Cucumber mosaic virus* under transmission electron microscope. CMV- CP gene was ligated in to pTZ57R/T vector and transformed to *E. coli* strain DH5α. The CMV-CP gene was sub cloned to the expression vector pET21a. The recombinant plasmid pET21a/ CMV- CP was transformed into *E. coli* strain Rosetta through heat shock procedure. Using IPTG (1 mM) the entire culture was induced and observed the CMV- CP under electron microscope for structural analysis.

Gulati *et al.* (2016) conducted structural study on *Tobacco streak virus* coat protein. Unlike other *Bromovidae* members, *Ilarvirus* particles are more liable and pleomorphic, has difficulties in their crystallization. *Tobacco streak virus*-CP gene was cloned in *E. coli* (DE3) BL21/pRSET- C. The protein was purified using sucrose density gradient ultra-centrifugation. The cells were lysed by sonication using the buffer containing 50 mM Tris (pH 8.5), 200 mM NaCl, 10 mM β-Mercapto-ethanol (β-ME), one per cent Triton-x100, 10 mM $CaCl_2$, PMSF (1 mM) and $ZnCl_2$ (1 mM). Virus like particles (VLPs) were pelleted from the clear lysate by ultra-centrifugation at 87,000 Xg for three hours. Re suspended the pellet containing VLPs in minimum volume of buffer containing 50 mM Tris (pH 8.5), 200 mM NaCl, 10 mM β-ME and $ZnCl_2$ (1 mM). Again ultra-centrifugation was conducted at 235,000 Xg for two hours. Purity of the proteins obtained was examined by 12 per cent SDS- PAGE.

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Sabharwal (2017) conducted an experiment to study the structure and function of *Pepper vein banding virus* encoded proteins. Using pET20b/ PVBV-CP as template, the author amplified PVBV- CP gene and ligated the amplicon to double digested pRSET- C vector. Positive clones were selected and overexpressed in *E. coli* BL21 (DE3)pLysS competent cells. The molecular mass of expressed coat protein in SDS- PAGE was slightly higher than that of the theoretical molecular weight (*i.e.*, 32 kDa) due to addition of amino acid sequences, including histidine hexamer. The purification of PVBV- CP was done through sucrose density gradient centrifugation.

2.7.2. Protein expression and purification for antiserum production

The expression of viral coat protein in *E. coli*, followed by purification and polyclonal antiserum production had been reported for a number of plant viruses. These antisera had been used successfully used for plant virus detection (Hema *et al.*, 2003).

The coat protein of *Banana bract mosaic virus* (BBrMV) was expressed in *E. coli* as fusion protein and was used to produce a high-titre BBrMV-specific polyclonal antiserum for serological assays (Rodoni *et al.*, 1997).Wanitchakorn *et al.* in 1997 ligated BBTV DNA-3 into prokaryotic expression vector (pMAL-c2). Coat protein was expressed and polyclonal antiserum was raised in rabbit and detected the virus even in asymptomatic plants.

Developing high quality antibodies to *Grapevine leafroll associated closterovirus*-3 (GLRaV-3) and other GLRaVs, are difficult because yield of purified viruses was low. Polyclonal and monoclonal antibodies specific to GLRaV-3 were developed from purified viral coat protein and effectively used in serological assays to detect GLRaV-3 in grapevine. GLRaV- 3- CP gene was cloned into the pRSET-C expression vector. The recombinant plasmid was transformed in *E. coli* BL21 (DE3) and expressed the capsid protein. The coat protein was purified by Ni-NTA affinity chromatography and raised antiserum in rabbit (Ling *et al.*, 2000).

Sugarcane streak mosaic virus (SCSMV), belong to *Potyviridae*, causes mosaic disease of sugarcane. The coat protein gene of Andhra Pradesh (AP) isolate of SCSMV (SCSMV-AP) was cloned on pRSET- A vector and expressed in *E. coli.* BL21 pLysS, the recombinant coat protein was used to raise high quality antiserum. The CP antiserum was used to develop an IC-RT-PCR based assay for the detection and discrimination of SCSMV isolates in AP. The sensitivity of the IC-RT-PCR was compared with DAC-ELISA and DIBA. It was found to be more sensitive and thus used to detect the virus in sugarcane (Hema *et al.*, 2003).

Jawdah *et al.* in 2004, immuno- diagnosed *Prune dwarf virus* (PDV) using recombinant coat protein (CP) mediated antiserum. Coat protein gene of PDV was isolated and amplicon was obtained at 700 bp. Ligated PDV- CP gene to pGEM-T vector and the PDV coat protein sequence was analysed with NCBI accessions. Coat protein gene was sub cloned to pRSET vector and transformed the recombinant plasmid to *Epicurian coli* BL 21-Gold cells. Later, antiserum was raised and standardised through Dot blot immunoassays, Indirect ELISA (plate-trapped) and DAS-ELISA.

Dezfooli *et al.* (2016) expressed N-terminal His-tagged Nipah virus matrix protein (48 kDa) in *Spodoptera frugiperda*- 9 (sf- 9) cell line using baculovirus expression system. Metal ion affinity chromatography was used to purify the NiV M protein and the yield was high. The results of western blotting and ELISA proved the antigenicity of recombinant M protein.

Selvarajan *et al.* (2016) expressed, viral-associated protein (VAP) of *Banana streak virus* in *E. coli*, and polyclonal antibodies were raised against purified recombinant VAP fusion protein in rabbits. Specificity and sensitivity of antibodies were tested using western blot, immunosorbent electron microscopy (ISEM) and ELISAs. This was the first report on the production of polyclonal antiserum against recombinant VAP of BSV. This VAP-based immunodiagnosis

had its own role in quarantine, germplasm exchange and phytosanitary certification programmes.

Papaya ringspot virus coat protein (PRSV- CP) gene was amplified and to include 6X His tag at the N-terminus of the protein, the gene was cloned into the pRSET- B. The PRSV- CP/pRSET- B plasmid was transformed in *E.coli* DH5α for protein expression using IPTG. The expressed recombinant PRSV-CP was purified using Ni-NTA His-tag protein purification kit. Recombinant coat protein mediated polyclonal antiserum was raised in six month old female Newzealand white rabbit. Functionalised the electrochemical sensor surface with the anti-PRSV-CP antibody for sensitive detection of the PRSV-CP, which serves as a rapid, economical, easy-to-use, tool for the detection of plant pathogens from the field (Valekunja *et al.*, 2016).

Arumugam *et al.* (2017) cloned the BBTV coat protein (CP) gene to expression vector pET28a (+). Transformed the recombinant plasmid to *E. coli* BL21. The cells and induced by adding 1mM IPTG. The expressed CP has 19.5 kDa as documented in SDS- PAGE. Hence, it was concluded that expressed BBTV-CP could be further purified through Ni- column chromatography and later used to raise antibody to detect BBTV.

The coat protein gene of *Grapevine fanleaf virus* was expressed in the *E. coli* BL21 (DE3) using pET 28a as expression vector. Coat protein has a molecular weight of 42 kDa. After induction by IPTG (1 mM) at 37°C, sonication and high-speed centrifugation were used for cell disruption as well as inclusion body separation. Protein was purified and polyclonal antiserum was raised, used for immunodetection of the virus (Shibaei *et al.*, 2018).

2.7.2.1. Protein expression and purification of Cucumber mosaic virus for antiserum production

Khan *et al.*, (2012) amplified the CP gene of CMV by PCR. Ligated in bacterial expression vector pQE30, having IPTG-inducible T7lac promoter.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis resolved the expressed CP in 25 kDa. The recombinant CP fused with histidine tag was purified from *E. coli* using nickel nitrilotriacetic acid resin. Purified protein was injected in rabbit and the antiserum later used for virus indexing.

The complete coat protein gene of *Cucumber mosaic virus* (belonging to sub group 1A) was cloned to pET21- d(+) expression vector. After expressing His-tagged fused coat proteins in *E. coli* strain BL21 (DE3) by IPTG induction, purification was done through Ni-NTA affinity column chromatography. Purified CMV recombinant CP was used for antiserum production. The virus infected lily samples were tested using the polyclonal antisera by ELISA and western blotting assays. Immuno capture-reverse transcriptase- PCR was standardised using the polyclonal antiserum. This study concluded that, there were three CMV isolates belong to subgroup IA, with respect to their immunological reaction with CMV specific polyclonal antibody (Kim *et al.*, 2016).

Koolivand *et al.* (2017) pioneered the production of coat protein mediated antiserum for indigenous isolate of CMV (Isolate B13, AY871070). Coat protein gene of CMV was sub- cloned from pTZ57/ CMV- CP into pET21a expression vector, later transformed into *Escherichia coli* strain Rosetta and induced with IPTG. Expression of CMV- CP was examined in SDS- PAGE and confirmed through western blotting. Expressed protein was purified using T7- Tag affinity purification kit and used for raising polyclonal antiserum in mice. The raised antiserum was applied in serological and sero-molecular tests for virus indexing.

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Materials and Methods

3. MATERIALS AND METHODS

The present study on "Development of recombinant coat protein for immunodetection of *Cucumber mosaic virus* infecting banana" was carried out in the Department of Biochemistry, Indian Institute of Science, Banglore, Division of Plant Pathology, Banana Research Station, Kannara and Department of Plant Pathology, College of Horticulture, Vellanikkara during 2018- 2019. The details of materials used and methodology followed are described in this chapter.

3.1. COLLECTION OF SAMPLES

Banana leaves showing typical symptoms of *Cucumber mosaic virus* (KAU, 2016; Mujtaba, 2017) maintained in the insect proof net house, Banana Research Station, Kannara, Thrissur, Kerala and from the field were collected, Infected samples along with negative and positive controls were tested for CMV infection by Direct Antigen Coating Enzyme Linked Immuno-Sorbent Assay (DAC- ELISA) using antiserum purchased from National Research Centre for Banana, Trichy, Tamil Nadu.

3.2. DIRECT ANTIGEN COATING ENZYME LINKED IMMUNO SORBENT ASSAY (DAC- ELISA)

3.2.1. Chemicals and buffers used for DAC- ELISA

The chemicals and buffers used for DAC-ELISA are given in Tables 3.1 to 3.3.

Table 3.1. Composition of coating buffer (10X, pH 9.2)

Chemicals	Quantity
Na_2CO_3	15.9 g
$NaHCO_3$	29.3 g
$NaNO_3$	2.8 g
Distilled water	1000 ml

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Dissolved Na_2CO_3 and $NaHCO_3$ in 50 ml distilled water, later added $NaNO_3$, adjusted the pH and made up volume to 1000 ml.

Table 3.2: Composition of Phosphate Buffered Saline (10X, pH 7.4)

Chemicals	Quantity
NaCl	80.0 g
KH_2PO_4	2.0 g
Na_2HPO_4	2.0 g
Na_2HPO_4	11.6 g
KCl	2.0 g
Distilled water	100.0 ml

Table 3.3. Composition of Antibody diluent, blocking and substrate buffers

Buffer	Composition
PBS- T buffer	10X PBS buffer (100 ml) and Tween 20.0 (1 ml) detergent was added to 900 ml distilled water.
Blocking buffer	5.0 g SDM (Spray dried milk) to 100 ml PBS- T
Antibody diluent buffer/ Enzyme conjugate diluent buffer	Added 20.0 g PVP and 2.0 g of bovine serum albumin (BSA) to 1l PBS- T.
Substrate buffer (100 ml)	Mixed 9.7 ml diethanolamine in 50 ml DW, adjusted the pH to 9.8 with concentrated HCl. Stored in amber or brown coloured bottle
Substrate buffer solution	1.0 mg PNPP in 1ml of substrate buffer

3.2.2. Procedure of DAC- ELISA

The infected banana leaves were ground in 1X coating buffer (1:5 w/v) using sterile pre-chilled mortar and pestle. The sample was transferred to 1.5 ml microcentrifuge tube and centrifuged at 5000 rpm for 10 min. Dispensed 100 µl of sample to ELISA microtitre plate (96 wells, Tarson Product Pvt. Ltd.) containing100 µl of 1X coating buffer with two per cent Poly vinyl pyrrolidone (PVP) (100 µl) and incubated at 37°C for wash (1 h.). Washed the plate with three changes of PBS- T (Phosphate buffered saline- Tween^{R} 20) buffer three minute for each wash. Then, added 200 µl blocking buffer and incubated the polystyrene micotitre plate at 37°C (1 h.). The plate was washed with three changes of PBS- T buffer, then added 200 µl primary antibody (NRCB Trichy, 1: 2000V/V). Covered the plate with aluminum foil and incubated at 4°C, overnight.

Washed the plates with three changes of PBS- T buffer. Added 200 µl of anti- rabbit alkaline phosphatase conjugate (Sigma Aldrich USA, 1:10000 V/V) as secondary antibody solution and incubated at 37°C (2 h.). Washed the plate with PBS- T and added 200 µl of P- nitrophenylphosphate (PNPP) substrate solution to each well in micotitre plate. Incubated in dark at room temperature for 30 min. Measured the absorbance at 405 nm in an ELISA plate reader and compared the absorbance value of test sample with healthy samples.

3.3. ISOLATION OF TOTAL RNA FROM CMV INFECTED LEAVES

Total RNA was isolated from young banana leaves, which showed positive reading in DAC- ELISA. Isolated RNA was stored at - 20°C for further studies.

In order to isolate the total RNA from leaf samples prepared 0.1 per cent Diethyl pyrocarbonate (DEPC) water and treated the mortar and pestle with DEPC water and autoclaved.

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3.3.1. RNA isolation using QIAGEN^{R} RNeasy^{R} Plant Mini Kit (cat. Nos. 74903 and 74904)

Disrupted 100 mg of leaf samples that showed positive result in ELISA. Disrupted the leaves with mortar and pestle previously treated with DEPC water. The tissues were ground with liquid nitrogen and decanted the samples to pre-cooled microcentrifuge tube of 1.5 ml volume. Added 450 µl RLT buffer to 100 mg tissue and mixed thoroughly. Transferred the lysate to QIAShredder spin column (lilac) placed in a collection tube (2 ml). Centrifuged for two minutes at 10,000 rpm. Transferred the supernatant to a new microcentrifuge tube (Not supplied, DEPC treated) without disturbing the pellet. Then, 100 per cent ethanol (0.5 volume) was added to clear the lysate. Transferred the samples to RNeasy MiniSpin Coloumn (pink) in a 2 ml collection tube (supplied). Centrifuged for 15 sec. at 10,000 rpm. Discarded the flow through. Then, added with 700 µl RW1 buffer and centrifuged at 10,000 rpm for 15 sec. Added 500 µl RPE buffer to RNeasyspin column and centrifuged for 15 sec. at 10,000 rpm. Again, added 500 µl RPE buffer RNeasy spin column and centrifuged for at 10,000 rpm for two minutes. Placed RNeasyspin column in microcentrifuge tube (1.5 ml, supplied along with the kit). Added 30 µl RNase free water directly to the membrane. Centrifuged for one minute at 10,000 rpm to elute the RNA.

3.3.2. Agarose gel electrophoresis: The isolated RNA was electrophoresed at 1.2 per cent agarose gel at 85V

Reagents used in agarose gel electrophoresis

1. 50X TAE buffer (pH 8.0)
   Composition of of 50X TAE buffer is given below

Tris base	242.0 g (pH 8.0)
Glacial acetic acid	57.1 ml
500 mM EDTA	100.0 g

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The components enlisted above were mixed and dissolved in 1000 ml distilled water.

2. Ethidium bromide Stock

Preparation of ethidium bromide (EtBr) stock was carried out by dissolving 10 mg EtBr in 1 ml of distilled water.

Procedure of Agarose gel electrophorosis

Agarose (1.2 g) was dissolved in 1X TAE buffer (100 ml). When the agarose gel became cool, added 2.5 µl of ethidium bromide. Waited until the gel became cool. Running buffer (1X TAE Buffer)was prepared from 50X TAE buffer using 0.1 per cent DEPC treated water. Poured the agarose gel in casting tray and loaded the samples with 6X loading dye (Thermo fisher scientific). Electrophoresis was carried out at 85 V, until the dye reached $3/4^{th}$ of the agarose gel. The gel image was documented by BioRad Gel Doc Ez Imager.

3.4. FIRST STRAND COMPLIMENTARY DNA (cDNA) SYNTHESIS

The first strand cDNA of isolated RNA was synthesised by Thermo scientific RevertAid First Strand cDNA synthesis Kit (Cat. No. 00168871). Added following reagents listed in Table 3.4 to sterile nuclease free tubes and kept on ice.

Table 3.4. Preparation of template for cDNA conversion

Reagents	Component	Quantity
Template RNA	Total RNA	1.0 µl
Primer	Gene specific primer	1.0 µl
Water	Nuclease free water	10.0 µl

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Mixed the components in the tube gently and incubated at 65°C for 5 min in thermocycler and the tubes were later chilled on ice. Then following reagents were added.

5X reaction buffer	4.0 µl
Ribolock RNase inhibitor	1.0 µl
10 mM dNTP mix	2.0 µl
Reverted aid MuLV-RT (Moloney Murine Leukemia Virus- Reverse Transcriptase)	1.0 µl
Total volume	20.0 µl

Then, tube was incubated at 40°C for 60 min. At last the reaction was terminated by heating at 70°C for 5 min. The reverse transcriptase reaction product was stored at -20°C for PCR amplification.

3.4.1. Quantification of cDNA

Quantification of cDNA was carried out using Nano drop 2000/2000cc (Thermo scientific). The software icon in the desktop was selected and opened. Loaded 1 µl of blank (*i.e.*, distilled water) to the nanodrop reader in order to calibrate the software. Each sample (1 µl) was loaded to the nandrop reader. Obtained the data and saved in folder.

3.5 POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION OF COAT PROTEIN GENE

3.5.1. Primers

CMV- coat protein specific primer reported by Cherian *et al.* (2004) (Table 3.5) was used in RT- PCR to detect *Cucumber mosaic virus*.

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Table 3.5. CMV- coat protein gene specific primer and its sequence

Primer	Sequence (5'- 3')	Length (bp)
RP- CMV- F	CATCGACCATGGACAAATCTGAATCAAC	28
RP- CMV- R	CTCTCCATGGCGTTTAGTGACTTCAGCAG	29

3.5.2. Master Mix preparation (For 5 reaction)

Composition of master mix is given below. After preparing master mix, the tubes were placed in thermocycler (Eppendorf Mastercycler Gradient). The primers were diluted with distilled water (1: 10). The cDNA synthesised using commercially available kit was used as template for PCR amplification. cDNA template (2 µl) was added to 23 µl of master mix .The details of master mix for the PCR amplification is given in Table 3.6.

Table 3.6. Composition of master mix for PCR amplification of CMV- CP gene

Components	Quantity
Water	100.0 µl (20 µl x 5)
Taq buffer A with 10 mM MgCl2	12.5 µl (2.5 µl x 5)
10 mM dNTP set	1.25 µl each
• Primer (10 mM) Sense primer Anti-sense primer	 2.5 µl (0.5 µl x 5) 2.5 µl (0.5 µl x 5)
Taq Polymerase (3U/µl)	1.0 µl (0.2 µl x 5)

3.5.3. The programme for PCR

The entire programme for PCR is summarised in Table 3.7.

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Table 3.7. The thermal profile for PCR

Step	Temperature (°C)	Time period
Initial denaturation	94	3 min
*Denaturation	94	30 sec
*Annealing	70.6	45 sec
*Elongation	72	1.30 min
Final elongation	72	10 min
Hold	4	5 min

(30 cycles of denaturation, annealing and elongation)

3.5.4. Agarose gel electrophorosis

The stock buffer (50X TAE) and EtBr stock were prepared as mentioned in 3.3.2. Agarose gel (1.2 per cent) was prepared using running buffer (1X TAE buffer). After it became cool, added 2.5 µl EtBr. and allowed to set. Loaded the samples in casting tray along with 6X loading dye. Later, the samples were electrophoresed at 85 V until the loading dye reaches 3/4th of the agarose gel.

3.6. PCR PRODUCT PURIFICATION

The CMV- CP amplicon obtained through the amplification of cDNA using CMV- CP specific primer was purified Using GeneiPure^{TM} Quick PCR Purification Kit (Cat. No. 117309).

The total volume of PCR product was made up to 100 µl with binding buffer. Then, the product (100 µl) was mixed with binding buffer (500 µl). GeneiPure^{TM} column was placed in collection tube (2 ml) and centrifuged for one minute at 11000 rpm. Discarded the flow through. GeneiPure^{TM} column was washed with 500 µl wash buffer and centrifuged for one minute at 11000 rpm. The column was placed back in collection tube. Diluted one volume of wash buffer with four volume absolute ethanol (Added instantly). GeneiPure^{TM} column was washed with 700 µl wash buffer and spinned for one minute at 11000 rpm. The column was kept back to the collection tube and again centrifuged for two minutes at

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11000 rpm to remove traces of wash buffer. Incubated the column at 70°C for two minutes to remove the ethanol. Added 50 µl of elution buffer to the column and centrifuged for one minute at 11000 rpm to elute the PCR product. The sample was stored at -20°C.

3.6.1. Agarose Gel Electrophoresis: Section 3.5.4

3.7. CLONING OF COAT PROTEIN (CMV- CP) GENE IN pGEM- T VECTOR

3.7.1 Ligation of CMV- CP gene with pGEM- T plasmid

Preparation of ligation mixture

The reagents given below were mixed thoroughly for the preparation of ligation mixture (Table 3.8).

Table 3.8. Ligation mixture for CMV- CP cloning in pGEM- T vector

Reagents	Quantity (µl)
2X rapid ligation buffer	5.0
pGEM- T Easy vector	1.0
Purified PCR product	4.0
T4 DNA ligase	1.0

The mixture was centrifuged at 10,000 rpm for two minutes to settle the whole contents to the bottom of the tube, and the same was incubated at 4°C overnight.

3.7.2 Preparation of competent cells

The competent cells to transform the recombinant plasmid was prepared according to standard protocol given by Mandel and Higa (1970).

E. coli DH5α cells were inoculated to Luria Bertini (LB) broth and kept at 150 rpm at 37°C for three to four hours to obtain optimum optical density (OD). The culture was transferred to ice cold okridge tube and kept on ice for 20 min. to

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arrest the growth. The culture was centrifuged at 3500 rpm for 15 min. at 4°C. Discarded the supernatant and re-suspended the bacterial pellet in sterile ice- cold 0.1 M $CaCl_2$ (10 ml) and kept on ice for 20 min. later, centrifuged at 3500 rpm for 15 min. at 4°C. Discarded the supernatant and re-suspended the pellet in sterile ice cold 0.1 M $CaCl_2$ (2 ml) and kept the suspension on ice for 10 min. Then, the bacterial suspension was transferred to 1.5 ml microcentrifuge tube (700 µl each), and stored at -80°C in the presence of 20 per cent glycerol.

3.7.3. Transformation of ligated plasmid (pGEM- T/CMV- CP) in to competent *E. coli* strain DH5α

The ligated product (10 µl) was transformed in to competent cells (700 µl) and kept on ice for 40 min. and later kept at 42°C for 90 sec. in a water bath. It was again kept on ice for five minutes and then added LB broth (250 µl). The transformed colonies were incubated at 37°C for one hour at 150 rpm. Centrifuged the culture at 10,000 rpm for two minutes at 4°C and dissolved the pellet in little amount of supernatant.

3.7.3.1. *Blue- white screening of recombinant colony* (Hanahan, 1985)

3.7.3.1.1. Chemicals used

Stock of 5- bromo- 4- chloro- 3- indolyl- β- D- galactopyranoside (Xgal) and Isopropyl β-D-1-thiogalactopyranoside (IPTG) were prepared and stored at -20°C.

- Dissolved 20 mg Xgal in 1 ml Dimethyl sulfoxide (DMSO).
- Dissolved 100 mM IPTG in 1 ml distilled water.

3.7.3.1.2. Procedure of blue- white screening

The transformants were plated on LB- Agar /Ampicillin (50 µg/ml)/ Xgal (12 µl)/ IPTG (4 µl) medium and incubated at 37°C overnight. Blue and white colonies were observed. Individually selected each white colony and streaked on LB-Agar/Ampicillin (50 µg/ml)/ Xgal/IPTG medium and incubated at 37°C, overnight.

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3.8. CONFIRMATION OF CLONING (pGEM- T/CMV- CP)

3.8.1. Confirmation of ligation through colony PCR

The selected recombinants were confirmed through PCR amplification of white colonies with CMV- CP specific primer.

3.8.1.1. Master Mix preparation

Master mix for five reactions was prepared using the components given in Table 3.9.

Table 3.9. Master mix for colony PCR

Components	Quantity
Water	100 µl (20 x 5)
Taq buffer A	12.5 µl (2.5 µl x 5)
10 mM dNTP set	1.25 µl each
CMV- CP specific primer (10 mM)	
Sense primer } 1: 10 dilution	2.5 µl (0.5 µl x 5)
Anti-sense primer } 1: 10 dilution	2.5 µl (0.5 µl x 5)
Taq Polymerase (3U/µl)	1 µl (0.2 x 5)

3.8.1.2. Preparation of template for colony PCR

Selected moderate sized single white colonies from sub cultured plate and each was dissolved in autoclaved distilled water (20 µl). Kept the cell lysate in thermocycler at 98°C for two minutes. Later, the template (2 µl) was added to 23 µl of master mix.

3.8.1.3. Programme for PCR

The thermal profile for colony PCR using CMV- CP specific primer was standardised (Cherian *et al.*, 2004) and the PCR programme for amplification is given in Table 3.10.

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Table 3.10. Thermal profile for colony PCR

Step	Temparature (°C)	Time period
Initial denaturation	94	3 min.
Denaturation	94	30 sec.
Annealing	70.6	45 sec.
Elongation	72	1.30 min.
Final elongation	72	10 min.
Hold	4	4 min.

(NB: 30 cycles are there, from denaturation to elongation)

3.8.1.4. Agarose gel electrophoresis: Section 3.5.4

3.8.2. Confirmation of ligation (CMV- CP/ pGEM- T) through restriction digestion

3.8.2.1. Isolation of pGEM- T/CMV- CP plasmid for restriction digestion

The recombinant plasmid was isolated from *E. coli* DH5α cells by alkaline lysis method, proposed by Birnboim and Doly (1979).

E. coli DH5α/pGEM- T/ CMV- CP cells were inoculated in LB/Ampicillin (50 mg/ml) medium. Incubated the culture at 37°C, 150 rpm overnight. The cells were centrifuged at 10,000 rpm for five minutes at 4°C. The pellet was dissolved in 1.5 ml P_1 (Table 3.11) and vortexed thoroughly. Added freshly prepared 1.5 ml P_2 (Table 3.11), then mixed gently by hand. Added 2 ml P_3 (Table 3.11), kept at room temperature for five minutes or until the solution became clear. Centrifuged the tubes at 8000 rpm for 10 min. at 4°C. The supernatant was collected in 1.5 ml microcentrifuge tube and centrifuged at 10000 rpm for three minutes at 4°C. Collected 700 µl supernatant in 1.5ml tube and added 700 µl isopropanol, mixed well by end-to-end rotator. Kept at -20°C for 30 min. or until a white precipitate was visible. Centrifuged the tubes at 13000 rpm for 10 min. at 4°C and tap dried the pellet on tissue paper. The tubes were kept at 37°C for 30 min. or until the ethanol evaporated. Dispensed pGEM- T/ CMV- CP plasmid in 20- 30 µl distilled

water. The composition of reagents required for plasmid isolation is enlisted in Table 3.11.

Table 3.11. Composition of reagents for plasmid isolation

Reagents	Composition
Resuspension solution ($P_{1)}$	50 mM Tris (pH 8 adjust the pH by Con. HCl), 10 mM EDTA and 100 µg/ml RNase
Lysis solution ($P_{2)}$	1 per cent SDS and 0.2 N NaOH
Neutralising solution ($P_{3)}$	3 M Potassium acetate (pH 5.5)

3.8.2.2. *Agarose Gel Electrophorosis:*

The plasmids were electrophoresed in 0.8 per cent agarose gel with running buffer (1X TAE buffer) at 85 V. Running buffer was prepared by the same protocol mentioned in 3.5.4.

3.8.2.3. Preparation of restriction digestion mix

The recombinant plasmid was subjected to restriction digestion using *Eco*R1 enzyme. The reagents for restriction digestion is mentioned in Table 3.12.

Table 3.12: Reagents for preparation of restriction digestion mix

Reagents	Quantity (µl)
pGEM- T/ CMV- CP plasmid	5.0
*Eco*R1 enzyme	0.4
Buffer	1.0
Autoclaved water	3.6
Total volume	10.0

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The mixture was centrifuged at 10, 000 rpm for two minutes at 4°C, to settle the whole sample at the bottom of the tube. Incubated the mixture at 37°C for three hours and observed the gel profile as mentioned in section 3.5.4.

3.8.3. Sequencing of recombinant plasmid

The pGEM- T/CMV- CP plasmid, which was confirmed through colony PCR and restriction digestion using CMV- CP specific primer and *Eco*R1 enzyme respectively, was sent to Agrigenome, Kakkanad, Kochi. The sequence obtained was further analysed and homology of the best aligned sequence of the sample was compared with CMV- CP sample, using BLASTn. Aligned the sequence in Cluster Omega (http://www.ebi.ac.uk/Tools/msa/clustalo) and found out the conserved region of CMV- CP.

3.9. SYNTHESIS AND VALIDATION OF CMV- CP SPECIFIC OLIGONUCLEOTIDE FOR PROTEIN EXPRESSION STUDIES

Primer pairs containing recognition sites of restriction enzyme, along with CMV- CP specific sequences were designed (Thompson *et al.*, 1994). The designed primer was sent to Eurofins Genomics India Pvt. Ltd., Whitefield, Banglore for the synthesis.

3.9.1. Designing of CMV- CP specific primer

The sequence obtained from pGEM- T/ CMV- CP was analysed (Section 3.8.3), found out GC rich region for primer synthesis. Added recognition site of *Bam*H1 (*i.e.*, GGATCC) in the 3'end of forward primer and recognition site of *Nhe*1 (*i.e.*, GCTAGC) in the 5'end of reverse primer. Later, it was confirmed recognition sequence of *Nhe*1 and *Bam*H1 are not within the CMV coat protein gene sequence.

3.9.1.1. Dilution of designed primer for PCR

The designed primer was diluted for further studies. For single reaction, 20 p.mol. CMV- CP primer is required, hence, dissolved the lyophilised powder of

oligonucleotide CMV- CP gene specific primer in sterile distilled water (with respect to synthesis scale of the primer given by manufacture) *i.e.*, made up the concentration of oligonucleotide to one n.mol./ ml.

3.9.2. Standardisation of annealing temperature for designed primer

The standardisation of annealing temperature of designed primer was carried out using gradient PCR. Among the range of temperature, temperature showing maximum amplification at 750 bp was selected as annealing temperature of the primer.

All the components except annealing temperature was kept constant in PCR programme. In gradient PCR, gradient was kept as 2.5 and temperature was 53.1°C. Annealing temperatures *viz.*, 53.6°C, 54°C, 54.4°C, 55°C, 56.3°C, 57°C, 57.7°C and 58.2°C were used.

3.9.2.1. PCR amplification for standardisation of annealing temperature

Master mix (for single reaction) was prepared as follows and the reagents for the master mix preparation (Table 3.13) and thermal profile for PCR amplification of CMV- CP gene (Table 3.14) is listed below.

Table 3.13. PCR master mix for amplification of CMV- CP gene using designed primer

Reagents	Quantity
Water	20. 35 µl
Taq buffer A (10 mM)	2.5 µl
dNTP set	0.25 µl each
Sense primer Anti-sense primer (1:10 diluted)	0.5 µl 0.5 µl
Taq Polymerase (3U/µl)	0.25 µl

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Table 3.14. PCR programme for amplification of CMV- CP gene using designed primer

Step	Temperature (°C)	Time period (Min)
Initial denaturation	95	5.0
*Denaturation	95	1.0
*Annealing	55	1.0
*Elongation	72	1.0
Final elongation	72	7.0
Hold	4	

(* 30 cycles of denaturation, annealing and elongation)

3.10. PCR AMPLICATION OF CMV- CP GENE USING DESIGNED PRIMER FOR PROTEIN EXPRESSION STUDIES

Pfu DNA polymerase was the enzyme used to amplify the gene of interest during PCR.

Table 3.15. Composition of master mix

Reagents		Quantity
Water		68 µl
5X HF Phusion buffer		20 µl
10 mM dNTP set		2.0 µl each
Sense primer	Primer (10 mM)	2.0 µl
Anti-sense primer		2.0 µl
Pfu DNA polymerase		1.0 µl
Dimethyl sulfoxide (DMSO)		3.0 µl

Preparation of template was carried out by dissolving pGEM- T/CMV- CP plasmid (1 µl) in sterile distilled water (10 µl). The template (2 µl) was added to 50 µl of master mix. Composition of master mix (Table 3.15) and thermal profile for PCR amplification are given in Table 3.16.

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Table 3.16: Different steps of PCR for amplification of CMV- CP gene

Step	Temparature (°C)	Time period
Initial denaturation	98	1 min.
*Denaturation	98	30 sec.
*Annealing	55	30 sec.
*Elongation	72	25 min.
Final elongation	72	10 min.
Hold	4	5 min.

(* 30 cycles of denaturation, annealing and elongation)

3.10.1. Agarose Gel Electrophorosis: Similar protocol as in section 3.5.4.

3.11. PCR PRODUCT PURIFICATION

3.11.1. PCR product was purified using GeneiPure™ Quick PCR Purification Kit (Cat No. 117309) by the procedure mentioned in section 3.6.

3.11.2. Agarose Gel Electrophoresis: Similar protocol as in 3.5.4.

3.12. CLONING OF COAT PROTEIN GENE TO EXPRESSION VECTOR

3.12.1. Restriction digestion

Both purified PCR product and expression vectors *viz.*, pRSET- C (Appendix-1) and pET28a (Appendix-2) were subjected to restriction digestion using *Bam*H1 and *Nhe*1 enzymes. The reagents used for restriction digestion of CMV- CP amplicon (Table 3.17) and expression vectors (Table 3.18) are enlisted below.

Table 3.17: Reagents used for restriction digestion of PCR product

Reagents	Quantity (µl)
Purified PCR product	8.2
*Nhe*1	0.4

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*Bam*H1	0.4
Tango buffer	1.0
Sterile water	NIL
Total volume	10.0

Table 3.18: Reagents used for restriction digestion of expression vectors

Reagents	**Quantity** (µl)
pRSET-C or pET28a plasmid	6.0
Nhe1	0.4
BamH1	0.4
Tango buffer	1.0
Sterile water	2.2
Total volume	10.0

The restriction digestion mixture was kept at 37°C for three hours, then the plasmid was heat inactivated at 80°C for 10 min.

3.12.2. Ligation of CMV- CP gene with expression vectors

Prepared the ligation mixture as given below and incubated at 16°C in a water bath overnight. The composition of ligation mixture is given in the Table 3.19.

Table 3.19. Composition of ligation mixture

Reagents	**Quantity (µl)**
T4 DNA ligase	1.0
Ligase buffer	1.0
Digested PCR product	6.0
Vector (pRSET- C or pET28a)	2.0
Total volume	10.0

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Ligation mixture without PCR product was also kept as control to check the frequency of self-ligation.

3.12.3. Transformation of ligated product into competent *E. coli* DH5α cells

E. coli DH5α cells were made competent for the uptake of DNA fragment, using $MnCl_2$ and $CaCl_2$ as described by Sabharwal (2017).

3.12.3.1 Preparation of acid salt buffer (ASB): The composition of ASB is given in Table 3.20

Table 3.20. Composition of acid salt buffer

Chemicals	Quantity (g)
40 mM Sodium acetate	0.328
70 mM Manganese chloride	1.38
100 mM Calcium chloride	1.47

Mixed all chemicals in distilled water, using magnetic stirrer and adjusted the pH to 5.5 using acetic acid. The buffer was filter sterilised using 0.22 µm nylon filter, in a laminar air flow chamber. The buffer was stored at 4°C for use.

3.12.3.2. Preparation of competent cells

Single colony of *E. coli* DH5α was inoculated in 50 ml LB broth. Incubated in a shaker (150 rpm) at 37°C for overnight. Primary LB medium (2 ml) was added to 200 ml secondary LB medium (1 per cent tryptone, 0.5 per cent yeast extract and 1 per cent NaCl). The culture was incubated at 30°C, 150 rpm, till OD value reaches 0.4 at 600 nm. Later, chilled the culture at 4°C for one hour to arrest the growth of DH5α cells. The cells were harvested by centrifugation at 3000 rpm for 15 min. at 4°C. Discarded the supernatant immediately after centrifugation to avoid further contamination of pellet with supernatant. The cell pellets were re-suspended in ASB (30 ml), then kept on ice for 45 min. Centrifuged the ASB treated cell suspension at 3000 rpm for 15 min at 4°C.

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Discarded the supernatant and collected the pellet. Re-suspended the pellet in ASB (5 ml) containing 20 per cent glycerol was frozen by liquid nitrogen and stored at -80°C as aliquot (100 µl).

3.12.3.3. Transformation of ligated product (pRSET- C/ CMV- CP or pET28a/ CMV- CP) into competent *E. coli* DH5α cells

Transformation of ligated plasmid into *E. coli* DH5α cells was carried out by the protocol given by Studier and Moffatt (1986).

Ligated product (10 µl) was transformed into competent cells (100 µl) and kept on ice for 45 min at 4°C and later kept at 37°C for five minutes and again kept on ice for two minutes. Added LB broth (1 ml) and incubated at 37°C for one hour at 150 rpm. Centrifuged the culture at 10,000 rpm for four minutes at room temperature, dissolved the pellet in little amount of supernatant. The transformants of *E. coli* DH5α/pRSET- C/ CMV- CP on LB- Agar/ Ampicillin(50 µl/ml) medium and *E. coli* DH5 α/pET28a/ CMV- CP were plated on LB- Agar/ Kanamycin (25 µl/ml) medium.

The control ligation mixture was also transformed into *E. coli* DH5α cells to know the frequency of undesired recombination or self-ligation. The selection of positive clones was done as per standard protocols (Sabharwal, 2017).

3.12.4 Isolation of recombinant plasmids

Individually selected 16 medium sized colonies transformed with recombinant pRSET- C from the plate and inoculated each colony in LB/Ampicillin (50 µg/ml). In a similar way, cells that were transformed with recombinant pET28a vector were inoculated in LB- Agar/ Kanamycin (25 µg/ml) medium in culture vials (34 ml) or falcon tubes (50 ml) and incubated overnight at 150 rpm. Pelleted the cells from each vial at 10,000 rpm for seven minutes at 4°C and dissolved the pellet in 150 µl P_1 and vortexed, then freshly prepared 150 µl P_2 was added and then gently mixed by hand, then added 200 µl of P_3 and kept for five minutes or until solution became clear, then centrifuged at 8000 rpm for 10

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min. at 4°C and collected the supernatant in 1.5 ml collection tube. Again, centrifuged at 10,000 rpm for three minutes at 4°C and collected 700 µl supernatant in 1.5 ml tube and added 700 µl isopropanol. Kept on end to end rotor and incubated at -20°C for 30 min. or until a white precipitate was formed. Centrifuged at 13000 rpm for 10 min. at 4°C and tap dried the pellet in tissue paper. The tubes were kept in a 37°C incubator for 30 min. or until the ethanol evaporated. Dispensed the plasmid in 20- 30 µl sterile distilled water.

3.12.4.1. Chemicals for plasmid isolation: Section 3.8.2.1.

3.12.4.2. Agarose Gel Electrophorosis: Section 3.8.2.2

3.13 CONFIRMATION OF pRSET- C/ CMV- CP OR pET28a/ CMV- CP THROUGH PCR

3.13.1 Master mix preparation

The master mix of 100 µl was prepared by adding the following reagents listed in Table 3.21.

Table 3.21: Reagents for the preparation of master mix (100 µl)

Reagents	**Quantity**
Water	81.4 µl
Taq buffer A (10 mM)	10 µl
dNTP set	0.5 µl each
Sense primer Anti-sense primer } (1:10 diluted)	2.0 µl 2.0 µl
Taq Polymerase (3U/µl)	1 µl

The template (pRSET- C/CMV- CP and pET28a/CMV-CP) (0.4 µl) was added to 24.6 µl of master mix.

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3.13.2. Programme for PCR

The thermal profile of PCR amplification is given in the Table 3.22

Table 3.22. Step wise programme details for PCR

Step	Temparature (°C)	Time period
Initial denaturation	95	5.0 min
*Denaturation	95	1.0 min
*Annealing	55	1.0 min
*Elongation	72	1.0 min
Final elongation	72	7.0 min
Hold	4	

(* 30 cycles of denaturation, annealing and elongation)

3.13.4. Agarose Gel Electrophorosis: Section 3.5.4

3.14. SEQUENCING FOR CONFIRMATION OF CMV-CP CONSTRUCT IN EXPRESSION VECTOR

The recombinant plasmids *viz*., pRSET- C/ CMV- CP and pET28a/ CMV-CP were transformed into competent *E. coli* DH5α cells as mentioned in 3.12.3. The recombinant plasmid multiplied within the host cell and it was isolated by alkaline lysis method as in 3.12.4 and sent for sequencing. The CMV- CP clone in recombinant plasmid, was selected for protein expression analysis as it has shown maximum homology with NCBI deposited samples.

3.15. TRANSFORMATION OF pRSET- C/ CMV- CP PLASMID TO EXPRESSION HOST

The recombinant (pRSET- C/ CMV- CP) plasmid was transformed in to both *E. coli* BL21(DE3)pLysS and *E. coli* Rosetta(DE3)pLysS cells as per protocols given in section 3.12.3. The transformed cells were spread on LB-Agar/Ampicillin (50 µg/ml)/Chloramphenicol (34 µg/ml) medium. The recombinants were selected based on antibiotic resistance.

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3.16. STANDARDISATION OF OVER EXPRESSION OF COAT PROTEIN GENE

Over expression of CMV- CP gene in *E. coli* BL21(DE3)pLysS/pRSET-C/CMV- CP and *E. coli* Rosetta(DE3)pLysS/pRSET-C/CMV- CP cells was checked by addition of IPTG.

3.16.1. IPTG induction

Prepared LB/Ampicillin (50 µg/ml)/Chloramphenicol (34 µg/ml) medium (24 ml), and transferred the medium to four sterile culture vials (6 ml each) and inoculated *E. coli* BL21(DE3) pLysS /pRSET- C/CMV- CP and *E. coli* Rosetta (DE3) pLysS/ pRSET-C/ CMV- CP to LB/Ampicillin (50µg/ml)/Chloramphenicol (34 µg/ml) medium (6 ml) and incubated at 37°C, 30°C and 16°C temperature at 150 rpm shaker incubator for 4 h., 6 h. and 12 h. respectively in order to attain the OD value of 0.4 at 600 nm. From each vial, culture (2 ml) was kept aside as un induced sample (*i.e.*, control) and added 1mM IPTG to induce the remaining culture (4 ml). Kept both induced and un induced culture, at 30°C for six hours. Each vial was subjected to centrifugation at 13,000 rpm for three min. at 4°C and collected the pellet. The pellet obtained was dissolved in 200 µl sterile distilled water. The expression transformats, showing better over expression of CMV- CP gene was selected for further studies.

3.16. 2. Sonication

Sonication was carried out by ultra sound prob sonicator in a thermoflask containing ice cubes and water. Sonication was done for one min. at two sec. pulse on and three sec. pulse off mode with 35 per cent amplitude. Immediately after sonication, the tubes were kept on ice and stored at 4°C.

3.16.3. Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE)

The composition of 12 per cent SDS-PAGE gel is given in Table 3.23.

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Table 3.23: Composition of SDS- PAGE gel

Stacking gel	**Component**	**Quantity**
	Sterile distilled water	4.7 ml
	30% Acrylamide	1.17 ml
	Tris (pH: 6.8)	875.0 µl
	10% SDS	70.0 µl
	10% Ammonium per sulphate (APS)	70.0 µl
	Tetramethylethylenediamine (TEMED)	5.0 µl
Resolving gel	**Component**	**Quantity**
	Sterile distilled water	8.2 ml
	30% Acrylamide	10.0 ml
	Tris (pH: 8.8)	6.3 ml
	10% SDS	250.0 µl
	10% APS	250 µl
	TEMED	20.0 µl
Sealing gel	**Component**	**Quantity**
	Sterile distilled water	6.0 ml
	30% Acrylamide	6.0 ml
	10% APS	200.0 µl
	TEMED	15.0 µl

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Acrylamide (30 per cent) was prepared by dissolving 29 g acrylamide and 1 g N,N'-Methylene bis- acrylamide(light sensitive) in 100 ml distilled water using a magnetic stirrer. Sealing gel was casted first in the apparatus. This was followed by resolving and stacking gel, respectively. After settling of sealing gel, poured water to confirm proper sealing. In order to avoid oxidation, immediately after the casting of resolving gel, poured 70 per cent ethanol (2-3 drops) to the casting tray. The sample was loaded as per the standard protocols (Smith, 1984).

SDS- PAGE gel was run at 125 Volt, till the dye reaches the sealing gel. Taken out the gel with ambient care and orientation of the gel was confirmed by slight slanting cut at the left hand corner of the gel. Staining solution (100 ml) was added to the gel and kept overnight in rocker at room temperature. After staining, thoroughly washed gel with tap water (3- 4 turns). Added 100 ml of destaining solution and kept the gel in rocker (6- 8 h.) at room temperature.

3.16.3.1. Staining of SDS- PAGE gel

Staining of low molecular weight protein samples was done in SDS-PAGE gel using Coomassie Brilliant Blue R-250 (CBB R- 250) and the composition of staining as well as destaining solution are listed in Table 3.24

Table 3.24. Composition of staining and destaining solution of SDS- PAGE gel

Staining solution		**Destaining solution**	
Reagents	**Quantity**	**Reagents**	**Quantity**
Methanol	40 ml	Methanol	40 ml
Acetic acid	10 ml	Acetic acid	10 ml
CBB R- 250	0.1 g	Total volume	100 ml
Total volume	100 ml		

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3.17. OVER EXPRESSION OF COAT PROTEIN

Different steps in over expression of CMV- CP includes, selection of buffer, IPTG induction, sonication and Ni- NTA purification.

3.17.1. Determination of isoelectric point of CMV- CP and pH of buffer

The isoelectric point of recombinant protein was found out using ExPasy-Translate tool and Protparam software. The pH was estimated at three units away from the isoelectric point (Gulati *et al.*, 2016; Sabharwal, 2017).

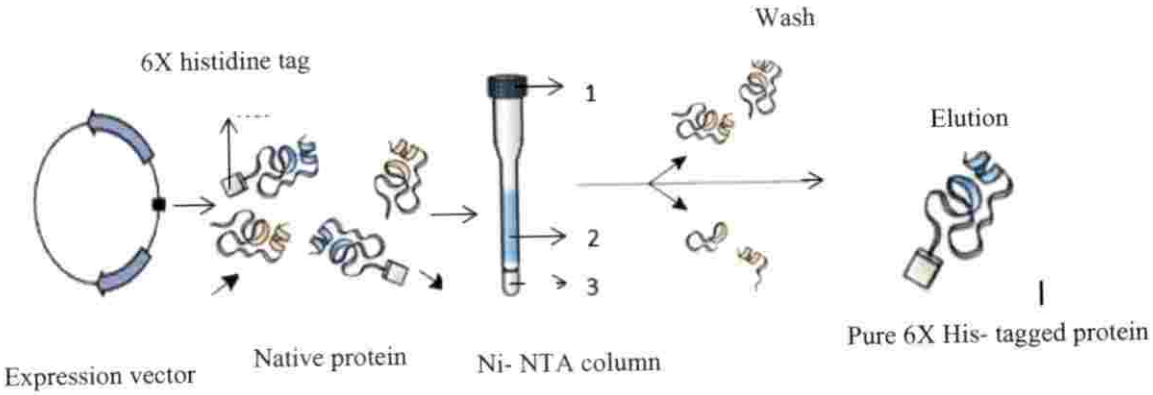
3.17.2. IPTG induction of *E. coli* BL21(DE3)pLysS/pRSET- C/ CMV- CP cells

E. coli BL21(DE3) pLysS/pRSET- C/CMV- CP cells were cultured in primary medium *i.e.*, LB/ Ampicillin (50 mg/ml)/Chloramphenicol (34 mg/ ml). Five millilitre of primary medium was then transferred to of secondary LB (12 g yeast extract, 6 g casein per 500 ml distilled water)/Ampicillin (50 µg/ml)/Chloramphenicol (34 µg/ml) medium (500 ml) and incubated the culture at 37°C overnight at 150 rpm. Later, 0.3 mM IPTG (from 1M IPTG stock) was added and incubated at 30°C, six hours at 150 rpm for induction. The culture was then centrifuged at 8000 rpm for 15 min. at 4°C. At last, the pellet was dissolved in minimum volume of sonication buffer and stored at -80°C.

3.17.3. Sonication

The pellet induced by IPTG was thawed and transferred to a plastic beaker and thoroughly dispensed in sonication buffer. Sonication was carried out in ice box, containing ice cubes and water at 30 per cent amplitude for five minutes at three sec. pulse on and six sec. pulse off using ultra sonic probe sonicator. The tubes were placed in ice immediately after sonication and stored at 4°C for further studies.

3.17.4. Ni- NTA (Nickel- Nitrilotriacetic acid) column purification



(Fig. 3.1: Flow chart of protein purification using Ni- NTA column chromatography; 1- Top lid of Ni- NTA column, 2- Resin and 3- Bottom lid of Ni- NTA column)

The entire protocol of Ni- NTA column purification is depicted in figure 3.1. After sonication, the culture was centrifuged at 10,000 rpm/ 10 min./4°C and collected the supernatant in 50 ml falcon tube and nickel beads (250 µl) were added to 50 ml of supernatant and kept at 4°C for 3.5 h, for binding of 6X histidine tag with nickel beads. Simultaneously, the column was set by wetting the membrane with tap water without allowing it to dry. Poured the supernatant, containing fusion protein bound with Ni beads to the column. Intermittent mixing was given by end cut micropipette (1 ml), then the flow through was collected by opening the bottom lid of the column. Washed the column using 10 ml wash buffer- 1, 30 ml of wash buffer- 2 (two wash, 15 ml each) and 10 ml of wash buffer-3, respectively.

Then, the recombinant protein was eluted using of elution buffer- 1 (1 ml). The bottom lid of column was closed for 10 min. (along with intermittent pipetting to prevent the sedimentation of beads) at 4°C, later collected the eluted fraction. Again, eluted the protein using of elution buffer- 1 (1 ml). Third elution was given by elution buffer- 2, following the previous step. After each elution, the protein was tested by Bradford reagent. The reagent (200 µl) was added to eluted protein (20 µl) and observed for the development of blue colour. Continued the

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elution using elution buffer- 2, until no colour change occurred to the Bradford reagent.

3.17.5 Buffers for protein purification

Buffers suitable for protein purification was selected as per Gulati *et al.* (2016). High concentration of imidazole is present in the elution buffer in comparison with wash buffer. The composition of sonication, wash and elution buffers are given in Table 3.25.

Table 3.25. Composition of sonication, wash and elution buffers

Buffer	Composition
Sonication buffer	20 mM Tris (pH: 8.0), 200 mM, NaCl, 1% Triton X100 and 20% glycerol
Wash buffer	
Wash buffer- 1	20 mM Tris (pH:8.0), 200 mM NaCl and 10 mM imidazole
Wash buffer- 2	20 mM Tris (pH:8.0), 200 mM NaCl and 20 mM imidazole
Wash buffer- 3	20 mM Tris (pH: 8.0), 200 mM NaCl and 50 mM imidazole
Elution buffer	
Elution buffer-1	20 mM Tris (pH:8.0), 200 mM NaCl and 150 mM imidazole
Elution buffer-2	20 mM Tris (pH:8.0), 200 mM NaCl and 300 mM imidazole

3.17.6. SDS- PAGE: Described in section 3.16. 3.

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3.17.7. Quantification of CMV- CP elutions

Quantification of CMV- CP was carried out using Nano drop 2000/2000cc (Thermo scientific). Selected the software icon and opened and loaded 1 µl of blank (*i.e.*, elution buffer or buffer in which the protein is dissolved) to the nano drop reader in order to calibrate the software. Then, each sample (1 µl) was loaded to the nano drop reader and recorded the value.

3.18. EVALUATION OF RECOMBINANT CMV COAT PROTEIN WITH COMMERCIALLY POLYCLONAL ANTISERUM IN ELISA

Direct antigen coating enzyme linked immune-sorbent assay was conducted to estimate the titre of CMV coat protein in the eluted fractions, using commercially available antiserum as described in section 3.2. Antiserum concentration was kept as constant (1: 2000, Mujtaba, 2017). The eluted protein was diluted with 1X coating buffer and dilutions *viz.*, 1: 10, 1: 200, 1: 500, 1: 1000, 1: 1500, 1: 2000 were prepared. Along with protein samples, one negative control and one positive control collected from the field were also used. Absorbance was measured using ELISA reader at 405 nm and compared values of each dilution with positive, negative and blank samples.

3.19. WESTERN BLOTTING USING CMV SPECIFIC ANTISERUM

3.19.1 SDS- PAGE: Described in section 3.16.3.

3.19.2 Buffers and chemicals

Composition of chemicals and buffers is given in Table 3.26.

Table 3.26. Composition of chemicals and buffers used in western blotting

Reagents	Composition
Transfer buffer (100 ml)	1X Tris- glycine buffer (from 5X Tris- glycine stock- 15 g tris of pH 8.3 and 72 g glycine/ 1l distilled water) and 20 ml methanol

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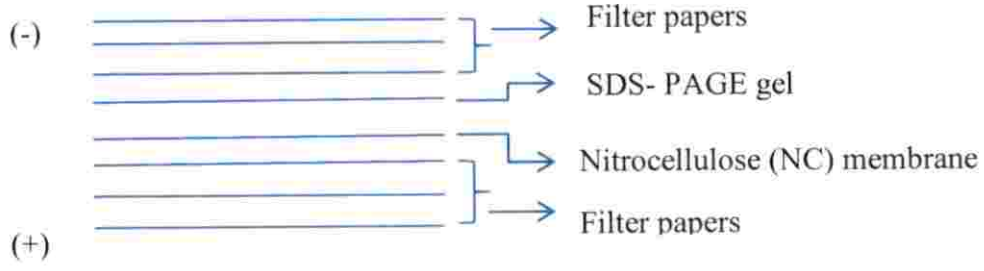
0.1%(w/v) Ponceau S Staining Solution	1 g Ponceau- S, 50 ml acetic acid and made up volume to 1 l with distilled water
Blocking buffer	Spray dried milk (SDM)/PBS buffer: 5 g SDM in 100 ml PBS
Primary antibody diluent buffer	20 µl of 1° antibody (CMV specific, polyclonal antibody,1: 500) dissolved in 10 ml PBS- T
Secondary antibody diluent buffer	6 µl of 2° antibody (Goat-anti-rabbit IgG, 1: 5000) dissolved in 30 ml SDM/PBS buffer

3.19.3. Procedure of immunoblotting

Filter papers and nitrocellulose membrane used for immunoblotting of coat protein were soaked in transfer buffer. Nitrocellulose membrane, filter paper and SDS- PAGE gel were kept for immunoblotting (3- 4 hours) at 80 mA, in a transfer apparatus in the presence of transfer buffer as depicted in figure 3.2. After immunoblotting, the NC membrane was stained with 0.1 %(w/v) Ponceau- S dye which is used for temporary staining. Protein bands were visualised on NC membrane in the presence of Ponceau- S dye and marked using a ball point pen. Washed off Ponceau- S dye with tap water until dye disappeared.



(Fig. 3.2 Arrangement of NC membrane, filter paper and SDS- PAGE gel in transfer apparatus)

3.19.4 Detection of antigen (*i.e.*, CMV coat protein)

Nitrocellulose membrane was incubated in blocking buffer for one hour at room temperature. Washed the membrane (two times, 5 min each) with 1X PBS.

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Added 15 ml of primary (1°) antibody diluent buffer and kept at 4°C overnight. Washed thrice with PBS- T and twice with 1X PBS, each for 10 min. in a high speed shaker. Secondary (2°) antibody diluent buffer (30 ml) was added to NC membrane and kept at room temperature for two hours. Washed thrice with PBS-T and twice with 1X PBS, each for 10 min. using high speed shaker. Development dye H_2O_2 (light insensitive) and luminol reagent (light sensitive) (250 µl each) were added and observed the bands.

3.20. WESTERN BLOTTING USING ANTI-HISTIDINE ANTISERUM

Followed the same procedure described in section 3.19 with the following changes.

Composition of antibody diluent buffers used in western blotting is given in Table 3.27.

Table 3.27. Antibody diluent buffers used in Western blotting

Antibody diluent buffer	**Composition**
Primary antibody diluent buffer	1 µl of 1° antibody (anti-histidine monoclonal antibody, 1: 5000) dissolved in 5 ml PBS- T
Secondary antibody diluent buffer	2 µl of 2° antibody (Goat-anti mouse IgG, 1: 5000) dissolved in 10 ml SDM/PBS buffer

3.21. SUCROSE DENSITY GRADIENT CENTRIFUGATION OF CMV- COAT PROTEIN

Sucrose density gradient centrifugation was used for the purification of CMV coat protein as per the protocol of Gulati *et al.* (2016) and Sabharwal (2017).

3.21.1. Culturing of *E. coli* (DE3) BL21 pLysS /pRSET- C/CMV- CP cells and induction by IPTG

The *E. coli* (DE3) BL21 pLysS/pRSET-C/CMV- CP cells were induced with IPTG and sonicated as mentioned in sections 3.17.2 and 3.17.3. The supernatant was then centrifuged using Beckman Coulter SW32 centrifuge (Figure.3.3) at 28,000 rpm for two and half hours at 4°C. The supernatant was discarded and the pellet was suspended in Tris- NaCl buffer of pH: 8.0 (1 ml) or SAT buffer of pH: 5.5 (1 ml) and kept at 4°C, overnight in end-to-end rotor.

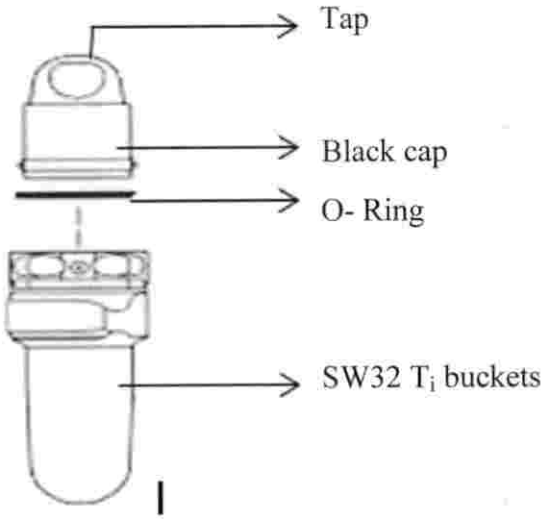

Fig. 3.3. Structure of Beckman Coulter SW32 Ti buckets

Composition and pH of buffers used for sucrose density gradient centrifugation is given in Table 3.28.

Table 3.28: Composition of buffers in sucrose density gradient purification

Buffer	pH	Composition
SAT	5.5	50 mM Sodium acetate and 0.02% Sodium thioglycolyte
Tris-Nacl	8.0	20 mM Tris (pH: 8.0) and 200 mM NaCl

3.21.2. Preparation of sucrose gradients

Prepared 40 per cent, 30 per cent, 20 per cent and 10 per cent sucrose solutions (20 ml each) in buffer (Table 3.28). Sucrose density gradient was prepared by gently pouring 40 per cent sucrose (8.5 ml), 30 per cent sucrose (7.5 ml), 20 per cent sucrose (6.5 ml) and 10 per cent sucrose (5.5 ml) to ultra-centrifuge tube in the respective order as depicted in Figure 3.4. Without disturbing the sucrose gradient, kept the tubes at 4°C for 12 h.

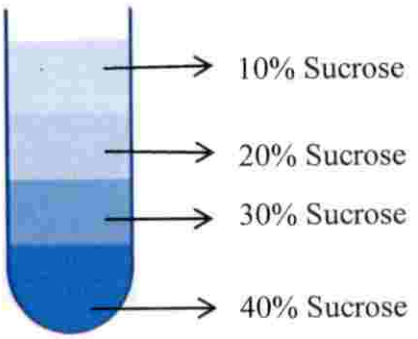

Fig.3.4 Arrangement of sucrose density gradient in an ultra-centrifuge tube

3.21.3. Ultra-centrifugation of coat protein in sucrose gradient

Installed the rotor body (SW 32) on the drive hub of ultra-centrifuge. Slowly turned the rotor to the right or clockwise to make sure that it was properly seated. Ultra-centrifugation tubes were inserted to Beckman Coulter SW32 T_i buckets. Ensured that bucket gaskets were tightly closed and evenly coated with vacuum grease. The ultra-pellet dissolved in suitable buffer (Table 3.28) was centrifuged at 10,000 rpm for15 min. at 4°C and collected the supernatant. The supernatant was transferred to sucrose gradient and ultra- centrifuged the gradient in Beckman Coulter SW32 rotor at 26,000 rpm for three hours at 4°C along with balance, which was prepared by the same buffer in which the ultra-pellet was dissolved. After centrifugation, a capillary tube was gently inserted in to the ultra-centrifugation tube. The fractions of sucrose gradient were collected from bottom of the tube in 1.5 ml microcentrifuge tube, using capillary suction. Each fractions

were labelled properly according to their respective concentration of sucrose gradient.

3.21.4. SDS-PAGE: The samples were electrophoresed in SDS-PAGE as mentioned in section 3.16.3.

3.21.5. Ultra-pelleting

Fractions showing maximum concentration of CMV- CP were pooled and ultra-centrifuged at 26,000 rpm for three hours at 4°C.The ultra-pellet was dissolved in respective buffer and loaded on SDS- PAGE (Plate 14C).

3.22. PURIFICATION OF CMV- CP PROTEIN THROUGH COMMERCIALLY AVAILABLE KIT

Attempt was made to purify CMV-CP through His60 Ni Gravity Columns (Cat. No. 635657) purification kit. Pre-packed His60 Ni Gravity columns (containing 1 ml resin) and buffers needed for protein extraction and purification was provided in the kit. The *E. coli* BL21(DE3)pLysS/pET28a/CMV- CP cells were used for protein production using commercially available kit.

3.22.1: Details of buffers available in commercial kit

Details of buffers in commercially available kit is enlisted in Table 3.29.

Table 3.29. Buffers available in commercially available protein purification kit

Buffers	Compostion
Equilibration Buffer	50 mM sodium phosphate, 300 mM sodium chloride, 20 mM imidazole; pH 7.4
Wash Buffer	50 mM sodium phosphate, 300 mM sodium chloride, 40 mM imidazole; pH 7.4. or 710 µl of His60 Ni Elution

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	Buffer with 9.29 ml of His60 Ni Equilibration Buffer
Elution Buffer	50 mM sodium phosphate, 300 mM sodium chloride, 300 mM imidazole; pH 7.4

3.22.2. IPTG Induction of *E. coli* BL21(DE3)pLysS/pET28a / CMV- CP/ cells

E. coli BL21(DE3)pLysS pET28a /CMV- CP cells were induced and cultured in three litres of LB/Ampicillin (50mg/ml)/Choramphenecol (34 mg/ ml) medium with 0.3 mM IPTG (Section 3.17.2).

3.22.3. Sample preparation for protein purification

His60 Ni X Tractor Buffer (2 ml/ 100 mg) was added to the *E. coli* BL2 (DE3) pLysS/pET28a/ CMV- CP cell pellet. Suspended the pellet in the buffer by pipetting. DNase- I was added to the suspended pellet at the rate of one micro litre per two millilitre of extract and mixed gently. The suspension was incubated on ice with intermittent mixing for 15 min., in order to avoid sedimentation. Centrifuged at 9500 rpm for 20 min at 4°C. Collected the supernatant carefully and discarded the pellet.

3.22.4. Protein purification using Ni- NTA Column

Equilibrated the column (1 ml) and all buffers to working temperature. The purification of CMV coat protein was carried out at 4°C. The matrix (agarose- coated Ni beads) was fully suspended before opening the column to prevent loss of resin that might have settled near the top cap (Figure 3.1).

The column was washed with five volumes of His60 Ni Equilibration Buffer. Put the bottom stopper on the outlet of the column. The sample was prepared as mentioned above. The clarified sample was added to the column and

carefully connected the top stopper. Target protein (*i.e.*, CMV- CP) was allowed to bind the Ni- coated agarose beads by slowly inverting the column for one hour at 4°C. The column was installed in a vertical position and allowed the resin to settle down. Carefully removed the top and bottom stopper and started collecting the fractions (1 ml). The column was washed with 10 volumes of His60 Ni Equilibration Buffer followed by 11 volumes of His60 Ni Wash Buffer. Target protein was eluted with approximately 10 volumes of Elution Buffer and collected the fractions (1 ml) in tubes. Bradford protein assay was carried out to monitor the elution of protein. The collected fractions were analysed by SDS-PAGE (3.16.3).

3.23 EVALUATION OF CMV- CP WITH COMMERCIALLY AVAILABLE ANTISERUM

Direct antigen coating enzyme linked immune-sorbent assay was carried out as per the procedure given in section 3.2. CMV coat protein was diluted with 1X coating buffer and dilutions (1:10, 1:100, 1: 200 and 1: 1000) were made. The dilutions, blank, positive and negative samples (from field) were also loaded in ELISA microtitre plate. The absorbance of each samples at 405 nm was recorded and compared the absorbance of the test samples with that of control and blank.

Results

4. RESULTS

The results of the investigation on 'Development of recombinant coat protein for immunodetection of *Cucumber mosaic virus* infecting banana' during 2018- 2019 are presented in this chapter under the following heads.

4.1. COLLECTION OF SAMPLES

Cucumber mosaic virus infected leaf samples were collected from Banana Research Station, Kannara, Thrissur, Kerala, based on the characteristic symptoms *viz*., younger leaves with parallel chlorotic streaks, distorted leaves with irregular wavy leaf margin along with necrotic tissues and dwarf plants which lag behind in growth, with narrow and small leaves (Plate 1).

4.2. DIRECT ANTIGEN COATING ENZYME LINKED IMMUNO SORBENT ASSAY

Along with healthy sample (negative control), the samples collected based on symptomatology of infectious chlorosis were tested using CMV specific antiserum (NRCB Trichy, 1: 2000V/V) and the following results were obtained. The sample collected from plant with dwarf stature and rosette appearance of leaves showed maximum absorbance at 405 nm followed by the one presented with parellel chlorotic streaks on leaves.

Even though samples KATC- 1 and KAPC- 1 showed symptoms of *Cucumber mosaic virus* infection, the absorbance was lesser than negative control at 405 nm. The results of DAC- ELISA is enlisted in Table 4.1.

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Healthy sample

KATC- 1

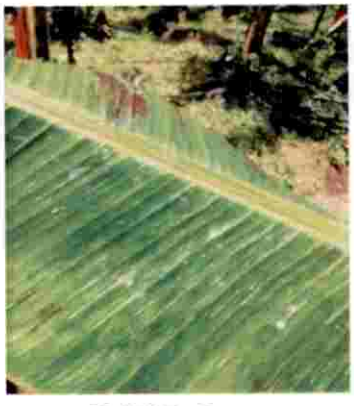
KAPC- 1

KAMC- 1

KAGC- 3

KANjC- 1

KANC- 4



KANC- 2

NDRNS- 4

Plate 1: Symptoms of *Cucumber mosaic virus* infection in banana leaves

Table 4.1. Absorbance value of test samples collected from field in DAC- ELISA

Sample	Variety	Absorbance at 405 nm
KANH- 1 (Negative control)	Nendran	0.099
KATC-1	Tjau lagada	0.093
KAPC1	Popoulu	0.074
KAMC- 1	Martaman	0.138
KAGC3	Grand- Nine	0.117
KANjC- 1	Njanlipoovan	0.105
KANC4	Nendran	0.156
KANC2	Nendran	0.144
NDRNS- 4	Nendran	0.205

Only the sample NDRNS- 4 showed positive reading in DAC- ELISA in comparison with negative control. Other samples like KANC- 2 and KANC- 4 showed higher absorbance value at 405 nm. Hence, NDRNS- 4, KANC- 2 and KANC- 4 were taken for further studies.

4.3. ISOLATION OF TOTAL RNA FROM CMV INFECTED LEAVES

Used QIAGEN^{R} RNeasyR Plant Mini Kit (Cat. Nos. 74903 and 74904).

Total RNA was isolated from CMV infected leaves, which were confirmed by DAC-ELISA. Electrophoresis of isolated RNA in 1.2 per cent agarose gel, showed two bands *viz.*, 18S rRNA and 28S rRNA. Maximum thickness of RNA band was observed for sample NDRNS- 4 followed by KANC- 2 (Plate 2A).

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4.4. FIRST STRAND cDNA SYNTHESIS

First strand cDNA was synthesised from total RNA using gene specific CMV primer as mentioned in materials and methods section 3.4. The results are documented in Table 4.2.

Table 4.2: Quantity of cDNA synthesised from total RNA of banana leaves

Sample	A_{260}/A_{280} ratio	Concentration (ng/ µl)
KANC- 4	1.68	1831.1
KANC- 2	1.69	1742.9
NDRNS- 4	1.71	2165.6

The A_{260}/A_{280} ratio of samples ranged between 1.68 to 1.71. Maximum concentration of cDNA was obtained from CMV isolate NDRNS- 4. Both CMV isolates KANC- 2 and KANC- 4 observed almost similar concentration of cDNA.

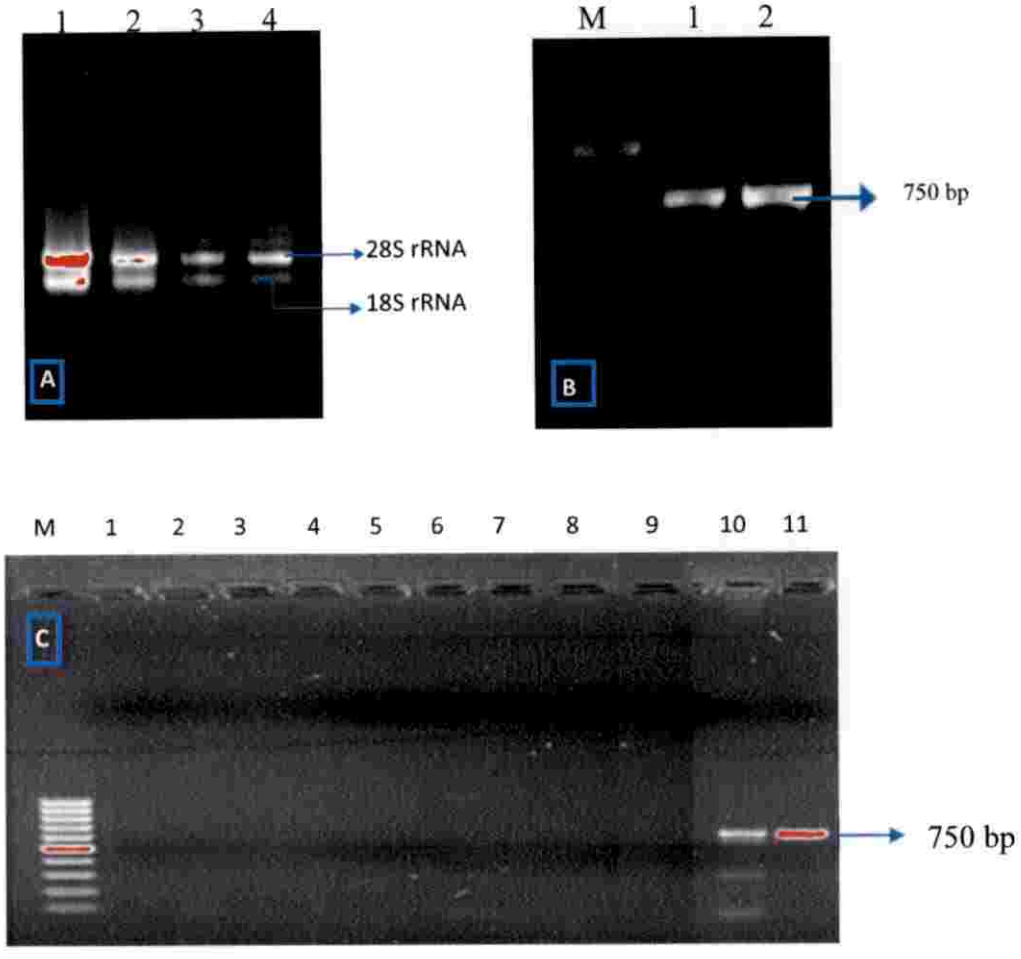
Samples NDRNS- 4, KANC- 2 and KANC- 4 were used for amplification of CMV- CP gene by RT- PCR.

4.5. POLYMERASE CHAIN REACTION FOR THE AMPLIFICATION OF CMV- CP GENE

The cDNA of KANC- 4, KACN- 2 and NDRNS- 4 were subjected to amplification using CMV- CP specific primer (Cherian *et al.*, 2004) and the amplicons were observed at 750 bp for KANC- 2 and NDRNS- 4. No amplification at 750 bp were observed for dilutions of 1:5, 1:10, 1:50 and 1:100 for both isolates KANC- 2 and KANC- 4 (Plate 2C).

4.6. PCR PRODUCT PURIFICATION

Amplified PCR products were purified GeniPureTM Quick PCR Purification Kit (Plate 2B). Based on the thickness of band observed for sample



A. Total RNA from banana leaves Lane M: Molecular marker (1 kb), Lane 1: Positive sample, Lane 2: NDRNS- 4, Lane 3: KANC2, Lane 4: KANC4 . **B**: PCR product purification Lane M: Molecular marker (1 kb), Lane 1: KANC- 2, Lane 2: NDRNS- 4 **C**. PCR amplification of CMV- CP gene Lane M: Molecular marker; KANC- 2 Samples: Lane 1- 1: 5, Lane 2- 1: 10, Lane 3- 1: 50, Lane 4- 1: 100 KANC- 4 Samples: Lane 5- 1: 5, Lane 6- 1: 10, Lane 7- 1: 50, Lane 8- 1: 100 Lane 9- KANC4, Lane 10- KANC2, Lane 11- NDRNS- 4

Plate 2: Molecular detection of *Cucumber mosaic virus* **A–** Total RNA from banana leaves **B-** PCR product purification **C-** PCR amplification of CMV- CP gene

loaded in 2nd lane, purified PCR product of NDRNS- 4 was taken for further studies.

4.7. CLONING OF COAT PROTEIN GENE TO pGEM- T VECTOR

The CMV- CP gene of NDRNS- 4 was ligated with pGEM- T vector and transformed into *E. coli* DH5α cells. Blue and white colonies of *E. coli* were observed on LB agar/Ampicillin/Xgal/IPTG medium. Assumed that white colonies are recombinants and they carry the construct of CMV- CP gene in pGEM- T vector, they were selected for further studies (Plate 3A).

4.8. CONFIRMATION OF pGEM- T/CMV- CP CLONING.

4.8.1. Confirmation of cloning through colony PCR

Colony PCR was carried out using CMV- CP specific primer. The samples loaded in lanes 2, 3 and 6 presented positive amplification at 750 bp, thus concluded that the white colonies obtained after blue-white screening carry the CMV- CP construct. Amplification at 750 bp was not observed in lanes 1, 4 and 5, which were loaded with blue colonies and transformation of blue colonies with non-recombinant plasmid was also confirmed (Plate 3B).

4.8.2. Confirmation of cloning through restriction digestion

4.8.2.1. Isolation of recombinant plasmid (pGEM- T/ CMV- CP) from E. coli **DH5α** *cells*

The recombinant plasmid (pGEM- T/ CMV- CP) was isolated by alkaline lysis method (Birnboim and Doly, 1979) from *E. coli* DH5α cells that were selected through colony PCR.

The increase in weight of recombinant plasmid (*i.e.,* 3.71 kb), due to the insertion of gene was observed in 0.8 per cent agarose gel. Three bands of plasmids are generally visualised in agarose gel *viz.*, linear, super coiled and circular (Plate 3C1).

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4.8.2.2. Restriction digestion of recombinant plasmid using EcoR1 enzyme

When the recombinant (pGEM- T/ CMV- CP) plasmid was digested with restriction enzyme *Eco*R1, the release of 750 bp CMV- CP amplicon from 3.0 kb pGEM- T vector was observed in 0.8 per cent agarose gel. The actual construct of CMV- CP in the recombinant plasmid has confirmed (Plate 3C2).

4.8.3. Sequencing of CMV- CP gene

The pGEM- T/ CMV- CP- NDRNS- 4 plasmid was isolated and sent to AgriGenome Labs Pvt Ltd. Kakkanad, Kochi, Kerala for sequencing. The CMV- Cp sequence obtained is given in Appendix- 1

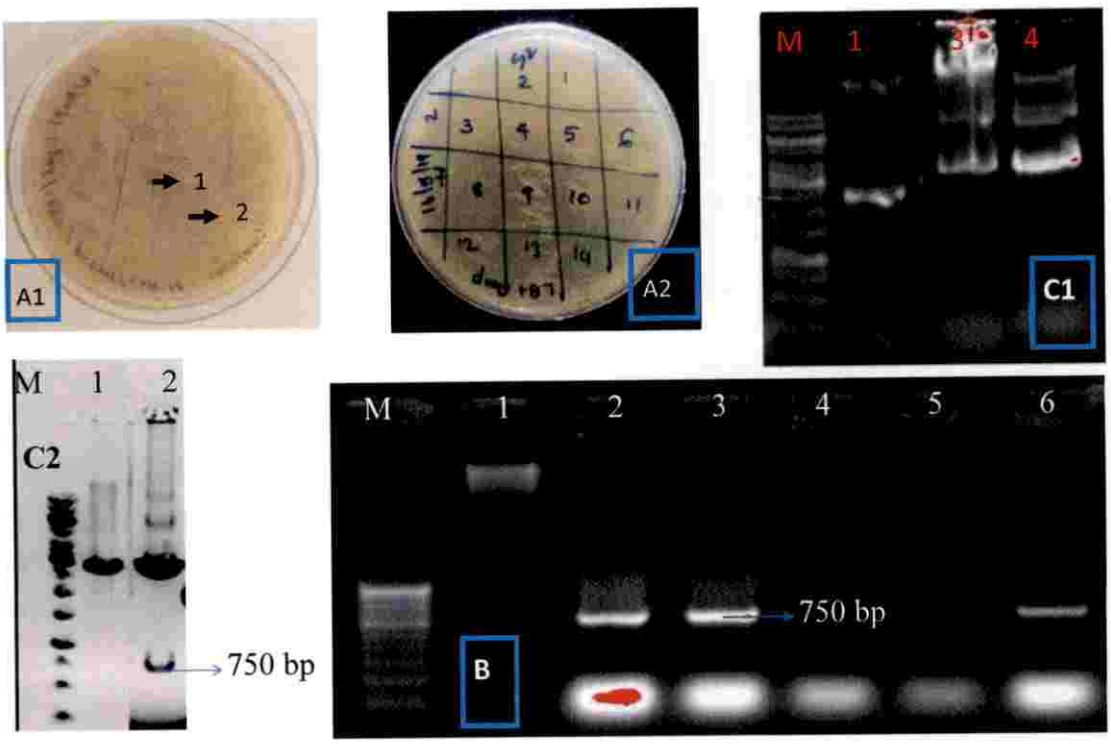
BLASTn analysis was done and compared the sequence with that of CMV- CP available in Genebank database

Comparison of CMV- CP nucleotide sequences showed 99.29 per cent similarity with accession MF280290.1 and 98.96 per cent similarity with accession AY125575.1 infecting banana. Both the accessions, showing maximum similarity with CMV- CP isolate NDRNS- 4, were also reported from Kerala, India (Plate 3D).

4.9. SYNTHESIS AND VALIDATION OF CMV-CP SPECIFIC OLIGONUCLEOTIDE FOR PROTEIN EXPRESSION STUDIES

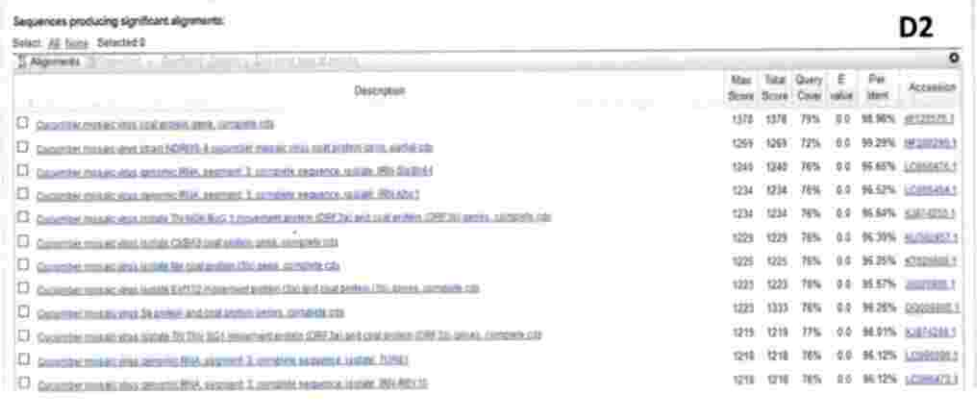
4.9.1. Designg of primer for expression analysis

Designed CMV- CP specific primers with recognition sites of restriction enzymes (*i.e.*, *Bam*H1 and *Nhe*1).The sequences of forward (30 bp) and reverse primers (28 bp) are given in Table 4.3 and the enzyme recognition sites are highlighted.



A- Blue- white screening of CMV-CP clones **A1**- Mother plate of blue- white screening **A2**- Sub culturing of white colonies 1- Blue colony, 2- white colony. **B**. colony PCR) Lane M- 1kb ladder, Lane 1, 4 and 5 - Non r- colonies, Lane 2, 3 and 6- CMV- CP recombinant **C**- Restriction digestion **C1**: Isolation of pGEM- T/ CMV- CP plasmid, Lane M: Molecular marker (1 kb), Lane 1: pGEM- T Easy plasmid; Lane 2 and 3: pGEM- T/ CMV- CP plasmid, **C2**: Restriction digestion of pGEM- T/ CMV- CP plasmid, Lane M: Molecular marker (100 bp), Lane 1: pGEM - T Easy vector, Lane 2: Digested pGEM- T/CMV- CP plasmid

Plate 3: Confirmation of CMV– CP in pGEM– T vector A– Blue and white colony screening **B–** Colony PCR **C**– Restriction digestion. D– Sequencing and analysis of CMV– CP gene



D– Sequencing and analysis of CMV– CP gene **D1**: CMV- CP gene alignment with NCBI deposited samples **D2**: CMV- CP sequence comparison with NCBI deposited samples

Plate 3: Confirmation of CMV– CP in pGEM– T vector, D- Sequencing and analysis of CMV– CP gene

Table 4.3. Sequences of designed CMV- CP specific primer

Forward primer (30 bp)	5'GGG **GCT AGC** ATG GAC AAA TCT GAA TCA ACC3'
Reverse primer (28 bp)	5'CCC **GGA TCC** TTA CTC TCC ATG GCG TTT AG 3'

4.9.2. Standardisation of annealing temperature for designed primer

Annealing temperature was standardised by gradient RT- PCR in Mastercycler gradient (Eppendorf) using pGEM- T/ CMV- CP plasmid as template. Primer annealing took place in all the selected temperatures. Highest intensity of amplification was observed at 55°C followed by 54.4°C, 57°C, 56.3°C, 57.7°C and 58.2°C. The lowest intensity was observed at 53. 6°C and 54°C. Annealing temperature of designed CMV- CP specific primer was selected as 55°C (Plate 4A).

4.10. PCR AMPLICATION OF CMV- CP GENE USING DESIGNED PRIMER FOR PROTEIN EXPRESSION STUDIES

The CMV- CP gene was amplified using designed primer and *Pfu* DNA polymerase. Amplicon was visualised at 750 bp in 1.2 per cent agarose gel. *Pfu* DNA polymerase is having high proof reading capacity. Since it is having high efficiency than *Taq* DNA polymerase, comparatively less time is required for polymerisation (Plate 4B).

4.11. PURIFICATION OF PCR PRODUCT

CMV- CP gene was amplified using designed primer and *Pfu* DNA polymerase enzyme. The amplicon at 750 bp was purified using GeniPure™ Quick PCR Purification Kit.

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4.12. CLONING OF COAT PROTEIN GENE IN EXPRESSION VECTOR

4.12.1. Restriction digestion

Both expression vector (pRSET- C and pET28a) and CMV- CP gene were digested at 37 ºC for three hours using restriction enzymes, *viz*., *Bam*H1 and *Nhe*1. The circular pRSET- C and pET28a vectors became linearize and sticky ends were developed on either sides of CMV- CP amplicon as well as the vector (Plate 4C1, 4C2 and 4C3).

4.12.2. Ligation of CMV- CP gene with expression vectors

The digested CMV- CP amplicon of isolate NDRNS- 4 was ligated to pRSET- C and pET28a vector as mentioned in 3.12.2.

4.12.3. Transformation of ligated product to *E. Coli* DH5α cells

Transformed the ligated product into competent *E. coli* DH5α cells as mentioned in 3.12.3. Colonies were observed on LB agar/antibiotic medium. Number of colonies were more on treatment plate in comparison with control, which indicated less frequency of self-ligation of expression vector (Plate 5A).

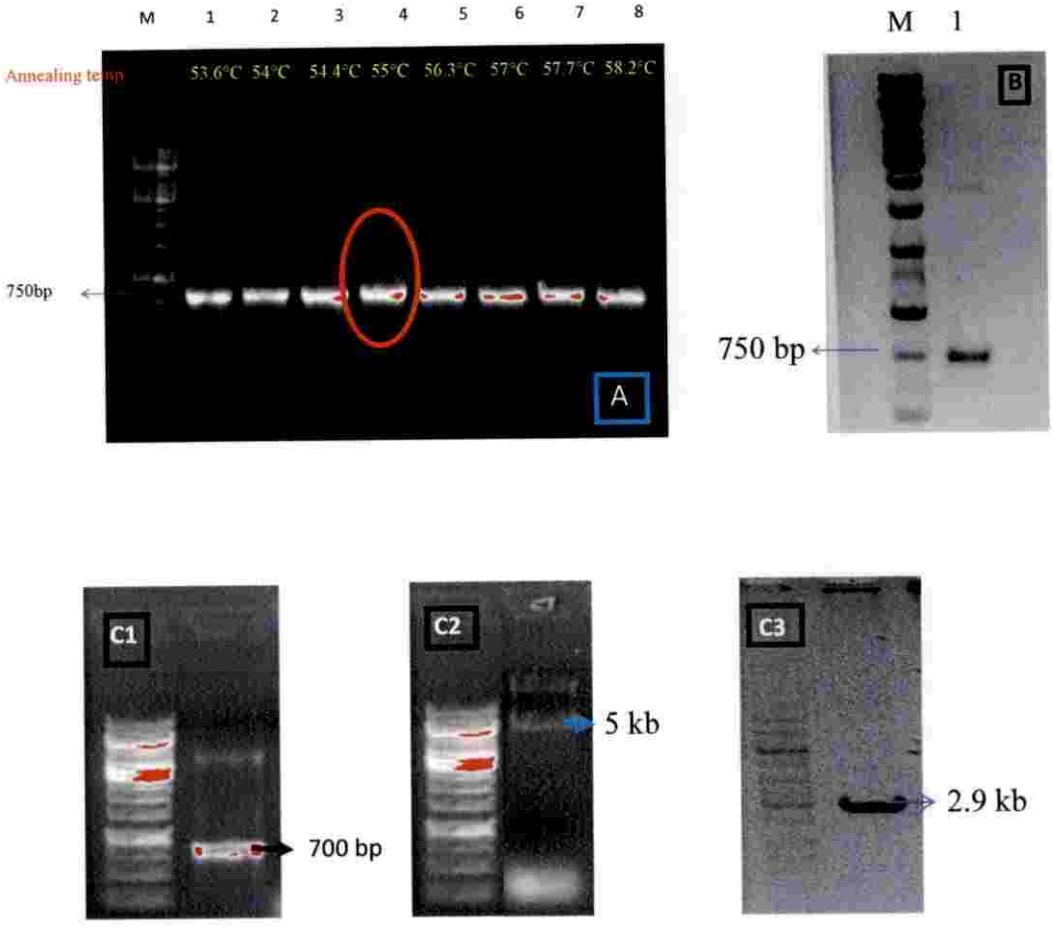
Compared to LB agar/ampicillin /pRSET- C/ CMV- CP/*E. coli* DH5α plate, LB agar/Kanamycin /pET28a/ CMV- CP/*E. coli* DH5α plate showed less number of colonies.

4.12.4. Isolation of recombinant plasmids

Plasmid was isolated from each *E. coli* DH5α cells and dissolved in 20 µl distilled water. Positive clone was selected based on the shift in 0.8 per cent agarose gel. The increased molecular weight of recombinant plasmid is due to the ligation of 750 bp CMV- CP amplicon (Plate 5B and 5C).

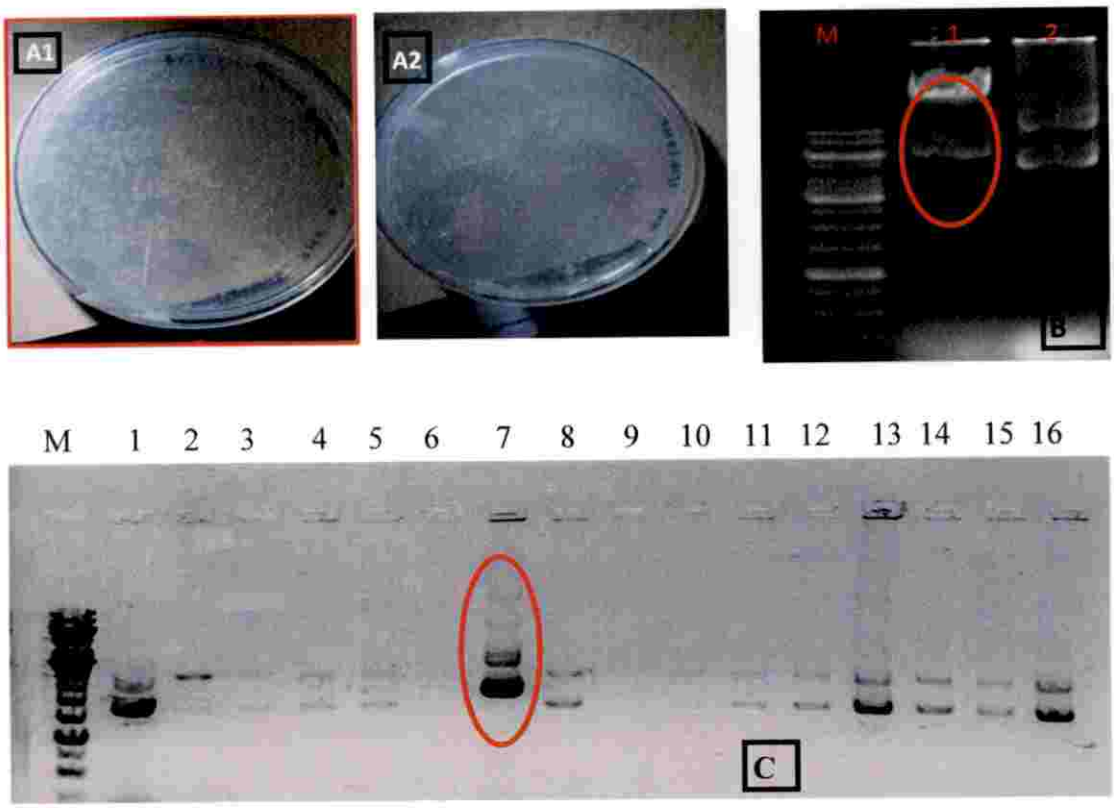
4.13. CONFIRMATION OF pRSET- C/ CMV- CP OR pET28a/ CMV- CP THROUGH PCR

Recombination in the plasmid (pRSET- C or pET28a) was confirmed through PCR using CMV- CP specific primer and *Taq* polymerase enzyme.



A. Standardisation of annealing temperature of designed CMV- CP primer **B.** PCR amplification of CMV CP using designed primer and *Pfu* DNA polymerase**, C-** Restriction digestion of CMV- CP amplicon and expression vectors using *Bam*H1 and *Nhe*1, **C1-** Restriction digestion of CMV- CP amplicon, Lane M: Marker, Lane 1: digested CMV- CP, **C2-** Restriction digestion of pET28a vector. Lane M: Marker, Lane 1: Digested pET28a, **C3-** Restriction digestion of pRSET- C, Lane M: Marker, Lane 1: digested pRSET- C vector

Plate 4: PCR amplification of CMV– CP gene using designed CMV– CP specific primer A– Standardisation of annealing temperature **B–** PCR amplification of CMV CP using designed primer and *Pfu* DNA polymerase **C-** Restriction digestion of CMV- CP amplicon and expression vectors using *Bam*H1 and *Nhe*1



A. pRSET- C/ CMV- CP plasmid in *E. coli* DH5α, **A1-** Treatment plate containing *E. coli* cells transformed with ligation mixture, **A2-** Control plate containing cells transformed with control ligation mixture, **B**: Selection of recombinant pET28a plasmid, Lane M: Molecular marker, Lane 1: Recombinant plasmid, Lane 2: pET28a **C-** Selection of recombinant pRSET- C plasmid, Lane M: Marker, Lane 1: pRSET- C plasmid, Lane 7: r- plasmid, Lane 2- 6 and 8- 16: non r-plasmid

Plate 5: Selection of positive clones of CMV– CP gene in expression vector

Amplicon of 750 bp was observed, in the lane, where recombinant plasmid was loaded and thereby confirmed the pET28a/CMV- CP or pRSET- C/ CMV- CP ligation (Plate 6A and 6B).

4.14. SEQUENCING FOR CONFIRMATION OF CMV-CP CONSTRUCT IN EXPRESSION VECTOR

The recombinant plasmids multiplied within the host cells and sequencing was done.

4.14.1. Sequencing of pRSET- C/ CMV- CP

Sequencing was done using designed primer (Table 4.3). The sequence obtained was analysed using NCBI BLASTn software. CMV- CP sequence was confirmed (Appendix 1). The sequence showed 98.88 per cent similarity with accession AY125575.1 and 98.22 per cent similarity with accession JF279609.1 of NCBI deposited sequences which were CMV- CP sequences of banana from Kerala (Plate 7A).

4.14.2. Sequencing of pET28a/ CMV- CP

Sequencing was done using designed primer as mentioned in section 4.14.1. and was confirmed. The sequence obtained was further analysed by NCBI BLASTn software and observed 94.33 per cent similarity with accession MF 280290.1 and 93.48 per cent similarity with accession JF279609.1 (Plate 7B).

Since, CMV- CP gene in recombinant pRSET- C plasmid showed maximum homology with NCBI deposited CMV- CP samples, recombinant pRSET- C vector was selected for further studies.

4.15. TRANSFORMATION OF pRSET- C/ CMV- CP PLASMID INTO EXPRESSION HOST

The recombinant plasmid was transformed into *E. coli* BL21(DE3)pLysS and *E. coli* Rosetta(DE3)pLysS cells for expression of CMV- CP gene according to the procedure described in 3.15.

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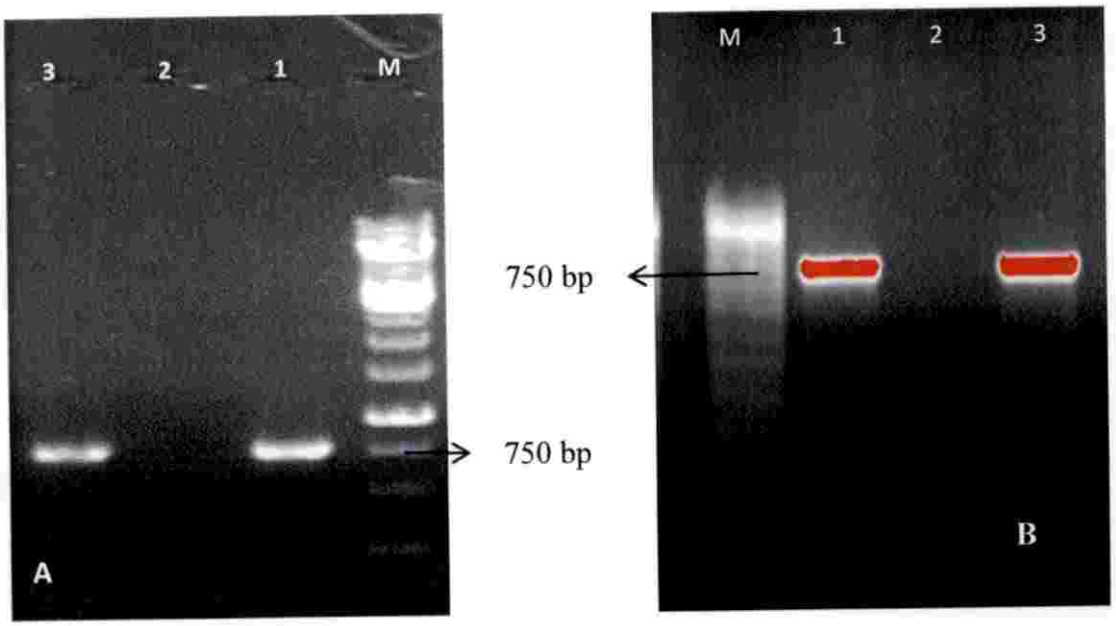
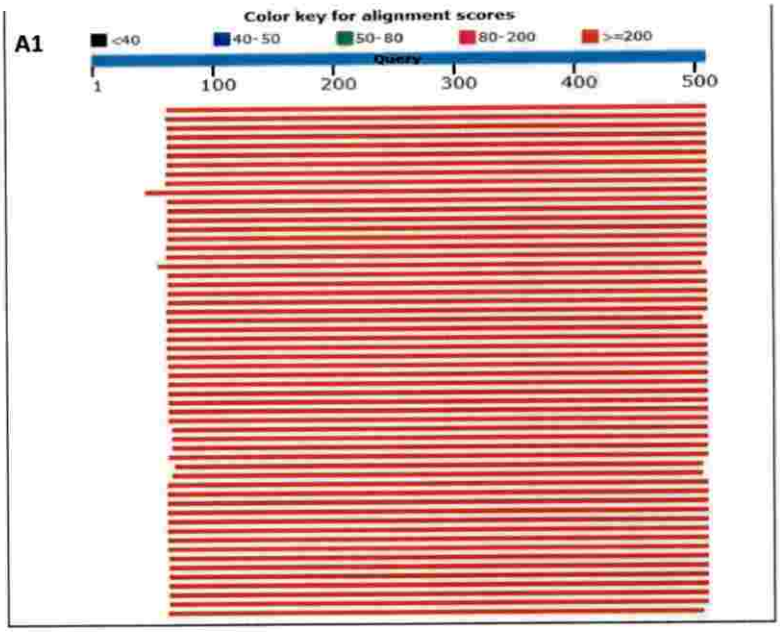


A. PCR amplification of pRSET- C/ CMV- CP, Lane M- Molecular marker, Lane 1- pGEM- T/ CMV- CP plasmid (Positive control), Lane 2- pRSET- C plasmid (Negative control), Lane 3- pRSET- C/ CMV- CP plasmid

B: PCR amplification of pET28a/CMV- CP, Lane M: Molecular marker (1 kb), Lane 1: pET28a/ CMV- CP , Lane- 2: Negative control (pET28a), Lane 3: Positive control (pGEM- T/ CMV- CP)

Plate 6: PCR amplification for confirmation of recombination in expression vector

A1



A2

Sequences producing significant alignments:

Select All None Selected 0

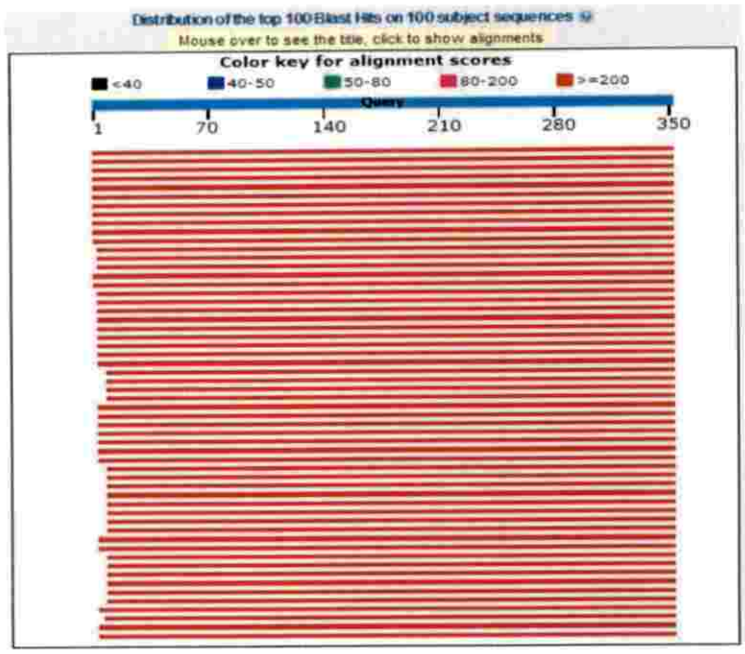
Alignments

Description	Max Score	Total Score	Query Cover	E value	Per Ident	Accession
Cucumber mosaic virus coat protein gene, complete cds	800	800	87%	0.0	98.88%	AY125575.1
Cucumber mosaic virus isolate Bakhi capsid protein (CP) gene, complete cds	785	785	88%	0.0	98.22%	JF279939.1
Cucumber mosaic virus isolate New Delhi chilli/pea coat protein gene, complete cds	784	784	87%	0.0	98.21%	MH513855.1
Cucumber mosaic virus isolate New Delhi ashgut coat protein gene, complete cds	778	778	87%	0.0	97.99%	MH513856.1
Cucumber mosaic virus isolate PAK-17-2 coat protein gene, partial cds	767	767	87%	0.0	97.54%	MF351523.1
Cucumber mosaic virus isolate Kerikeri Pea coat protein gene, complete cds	761	761	87%	0.0	97.32%	JX112091.1
Cucumber mosaic virus strain CMV-G10 coat protein gene, complete cds	761	761	87%	0.0	97.32%	AF541891.1

A- Confirmation of CMV– CP construct in pRSET– C vector, **A1-** BLAST analysis of CMV CP nucleotide sequence using NCBI database in pRSET– C/ CMV- CP plasmid, A2: CMV- CP gene alignment with NCBI deposited samples in pRSET– C/CMV- CP plasmid

Plate 7: Confirmation of recombination in expression vector, A: CMV– CP construct in pRSET– C vector

B1



B2

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
[illegible]	542	542	100%	4e-150	94.33%	[illegible]
[illegible]	525	525	100%	4e-145	93.48%	[illegible]
[illegible]	520	520	100%	2e-143	93.20%	[illegible]
[illegible]	520	520	100%	2e-143	93.20%	[illegible]
[illegible]	514	514	100%	1e-141	92.92%	[illegible]
[illegible]	514	514	100%	1e-141	92.92%	[illegible]
[illegible]	514	514	100%	1e-141	92.92%	[illegible]
[illegible]	508	508	100%	4e-140	92.63%	[illegible]
[illegible]	508	508	100%	4e-140	92.63%	[illegible]

B- Confirmation of CMV– CP construct in pET28a vector, B1- BLAST analysis of CMV CP nucleotide sequence using NCBI database in pET28a/ CMV- CP plasmid, B2: CMV- CP gene alignment with NCBI deposited samples in pET28a/CMV- CP plasmid

Plate 7: Confirmation of recombination in expression vector, B– CMV– CP construct in pET28a vector

4.16. STANDARDISATION OF OVER EXPRESSION OF COAT PROTEIN GENE

The expression systems were induced with 1mM IPTG at different temperature and time as mentioned in 3.16 and the expression of coat protein was evaluated in SDS- PAGE.

4.16.1. Standardisation of *E. coli* BL21 (DE3) pLysS /pRSET- C/CMV- CP for over expression

E. coli BL21(DE3) pLysS /pRSET- C/CMV- CP was induced as mentioned in section 3.16. The protein band of 25 kDa was present in all the combinations of temperature and time. Thus, the induction of CMV- CP gene in *E. coli* BL21 (DE3) pLysS was confirmed (Plate 8A).

4.16.2. Standardisation of *E. coli* Rosetta(DE3) pLysS /pRSET- C/CMV- CP cells for overexpression of CMV- CP gene

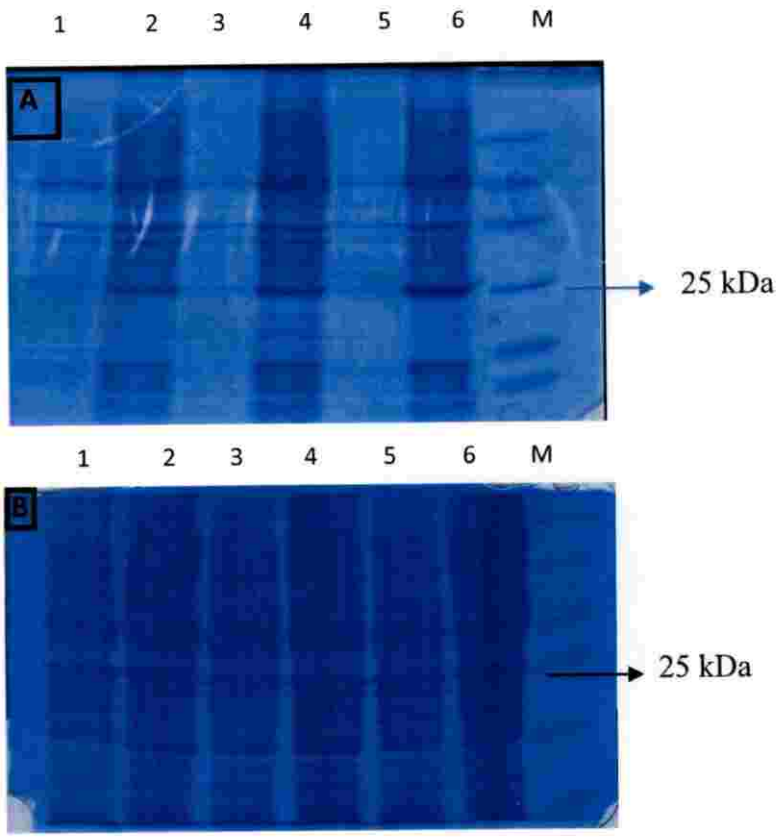
E. coli Rosetta (DE3) pLysS /pRSET- C/CMV- CP was induced as mentioned in 3.16. No difference was observed in protein profile between induced and un induced cells. Hence, concluded that CMV- CP gene cannot be over expressed in *E. coli* Rosetta (DE3) pLysS (Plate 8B).

4.17 OVER EXPRESSION OF COAT PROTEIN GENE

The coat protein was over expressed using 0.3 mM IPTG in selected buffer and purified through Ni- NTA column chromatography.

4.17.1. Determination of isoelectric point of CMV- CP and pH of buffer

CMV- CP sequence was translated to amino acid sequence using ExPASy- Translate tool (Plate 9).The amino acid sequence with highest ORF was selected and analysed through ProtParam. The theoretical pI of amino acid was found to be 10. 39. The pH of buffer suitable for solubilisation of CMV- CP was computed from the theoretical pI. *i.e.*, pH of buffer suitable for CMV- CP = (Theoretical pI of amino acid) +/- (3.0). Accordingly, the pH of elution buffer was fixed as 8.0.



A- Over expression of CMV– CP gene in *E. coli* BL21(DE3)pLysS cells, Lane M: Protein marker; Lane 1, 3 and 5: Un induced cultures (*E. coli* BL21 (DE3) pLysS/ pRSET- C/CMV- CP) kept at 16°C 30°C and 37°C for 12 h, 6 h and 4 h respectively; Lane 2, 4 and 6: Induced culture *(E. coli* BL21 (DE3) pLysS/ pRSET- C/ CMV- CP) at 16°C, 30°C and 37°C for 12 h., 6 h. and 4 h. respectively **B-** Over expression of CMV– CP gene in *E. coli* Rosetta(DE3)pLysS cells, Lane M: Protein marker; Lane 1, 3 and 5: Un induced cultures (*E. coli* Rosetta (DE3) pLysS/ pRSET- C/CMV- CP) kept at 16°C, 30°C and 37°C for 12 h., 6 h. and 4 h. respectively; Lane 2, 4 and 6: Induced culture *(E. coli* Rosetta (DE3) pLysS/ pRSET- C/ CMV- CP) at 16°C, 30°C and 37°C for 12 h., 6 h. and 4 h. respectively

Plate 8. Standardisation of over expression of CMV- CP in *E. coli*

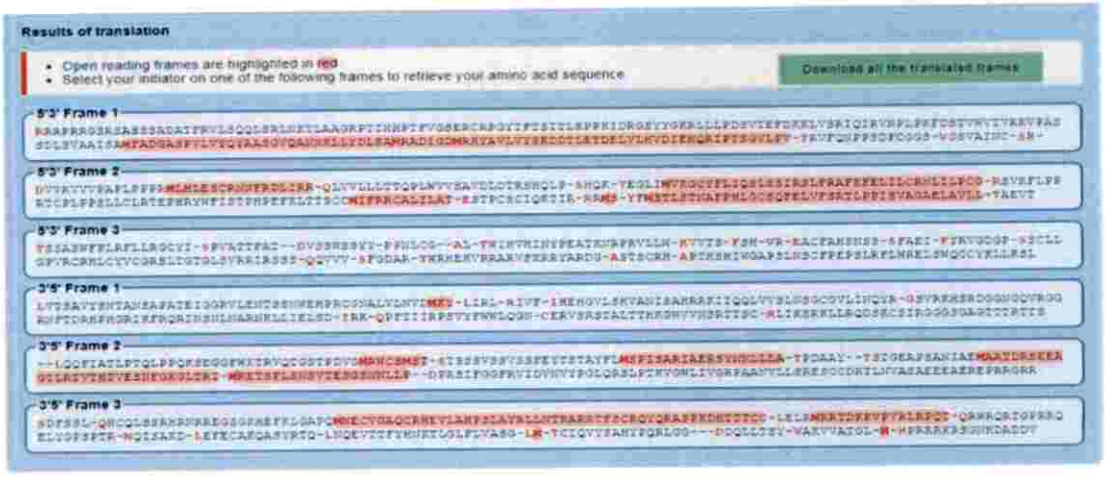


Plate 9. Translation of CMV- CP sequence using ExPASy- translate tool amino acid sequence with the marking is selected for ProtParam analysis

Based on the reports of Gulati *et al.* (2016), Tris- NaCl buffer of pH 8.0 was selected for protein extraction.

4.17.2. IPTG induction and sonication

The cells were induced with 0.3 mM IPTG and then sonicated in Tris- NaCl (pH 8.0) (Plate 10A) as explained in section 3.17.2 and 3.17.3.

4.17.3. Ni- NTA column purification of *Cucumber mosaic virus* coat protein

All fractions (*i.e.*, cell pellet, supernatant, flow through, wash and elution) were electrophoresed in SDS- PAGE gel (Plate 11A). The presence of 25 kDa protein was observed on SDS- PAGE in all the fractions except cell pellet, which implies the solubility of CMV coat protein in Tris- NaCl (pH 8) buffer. Out of the four elutions, maximum concentration of CMV- CP was observed in elution- 2 followed by elution- 3. Quantity of 25 kDa protein was very low in elution- 1 and 4. On reaction with Bradford reagent, the colour development was more in initial elutions than final elutions (Plate 11B).

4.17.4 Quantification of recombinant CMV coat protein by NanoDrop

The CMV- CP in eluted fraction was quantified as per the procedure explained in 3.17.7. The results are given in Table 4.4.

Table 4.4. Quantity of coat protein eluted through Ni- NTA column

Elution fraction	Quantity of protein (mg/ ml)
Elution- 1	0.129
Elution- 2	0.795
Elution- 3	0.686
Elution- 4	0. 626

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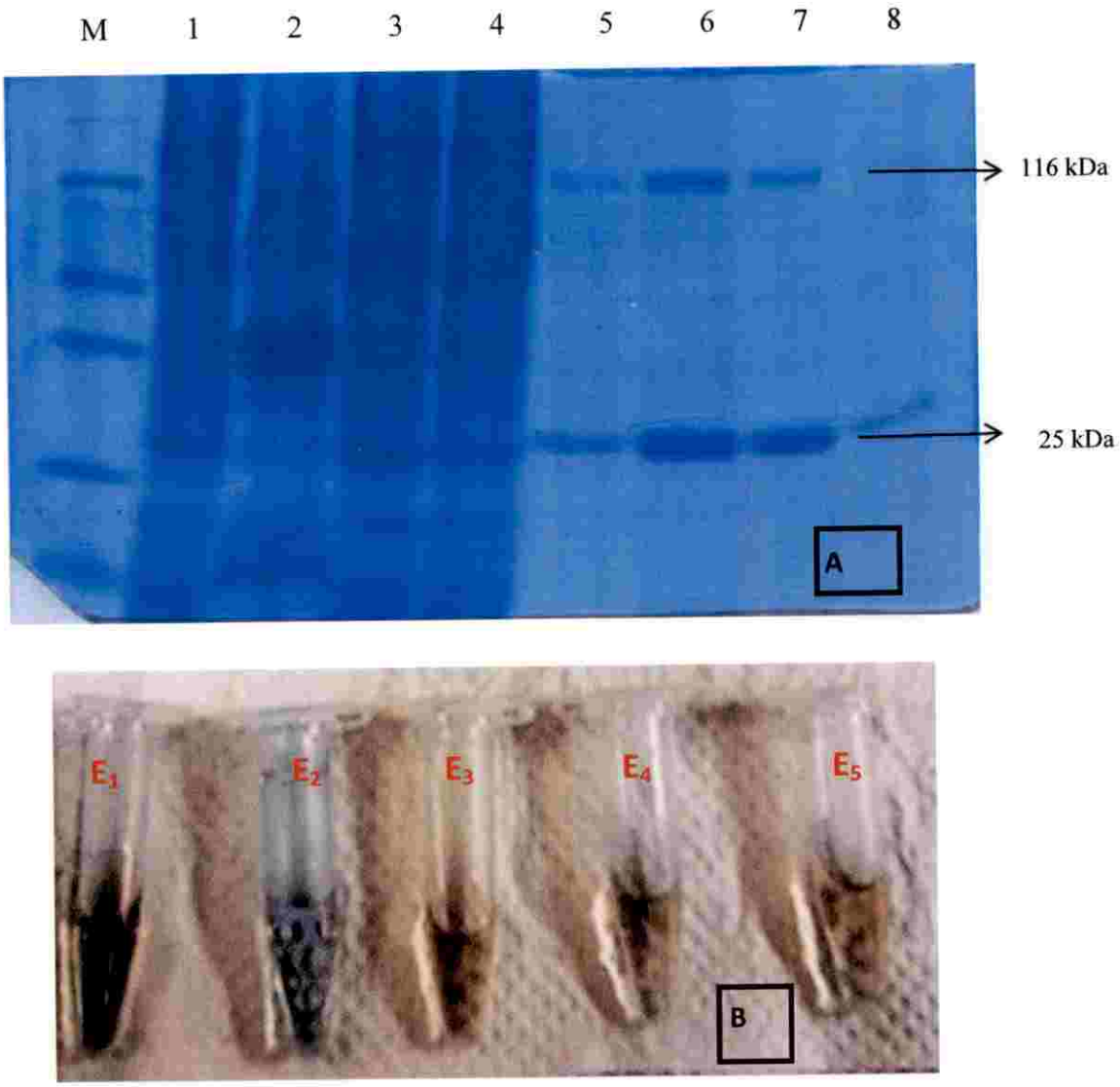
Pate 10. Purification of 6X His tagged CMV- CP though Ni- NTA column chomatography A– Sonication of bacterial suspension **B-** Ni– NTA column



A- SDS- PAGE gel of Ni- NTA purified CMV coat protein, Lane M- Marker. Lane 1- Supernatant; Lane 2- Pellet; Lane 3- Flow though, Lane 4- Wash, Lane 5- Elution 1, Lane 6- Elution- 2 Lane 7- Elution- 3 **B-** Testing the protein concentration of elutions of Ni- NTA column chomatography using Bradford reagent, E_1- Elution- 1; E_2- Elution- 2; E_3- Elution- 4; E_4- Elution- 5; E_5- Elution- 5

Plate11: Analysis of purified protein fractions

4.18. EVALUATION OF RECOMBINANT CMV COAT PROTEIN WITH COMMERCIALLY AVAILABLE POLYCLONAL ANTISERUM IN ELISA

Recombinant CMV coat protein was evaluated using commercially available CMV- CP specific polyclonal antiserum in DAC- ELISA as mentioned in 3.18. Out of the different dilutions of coat protein *viz.*, 1: 10, 1: 200, 1: 500, 1: 1000, 1: 1500 and 1: 2000, 1: 10 dilution of elution- 2 possessed maximum absorbance (0.428) at 405 nm. Positive sample recorded double the value of negative sample. The absorbance of all the dilutions was more than that of negative sample. (Table 4.5) (Plate 12A).

Table 4.5: Absorbance of CMV coat protein pRSET- C vector in DAC- ELISA

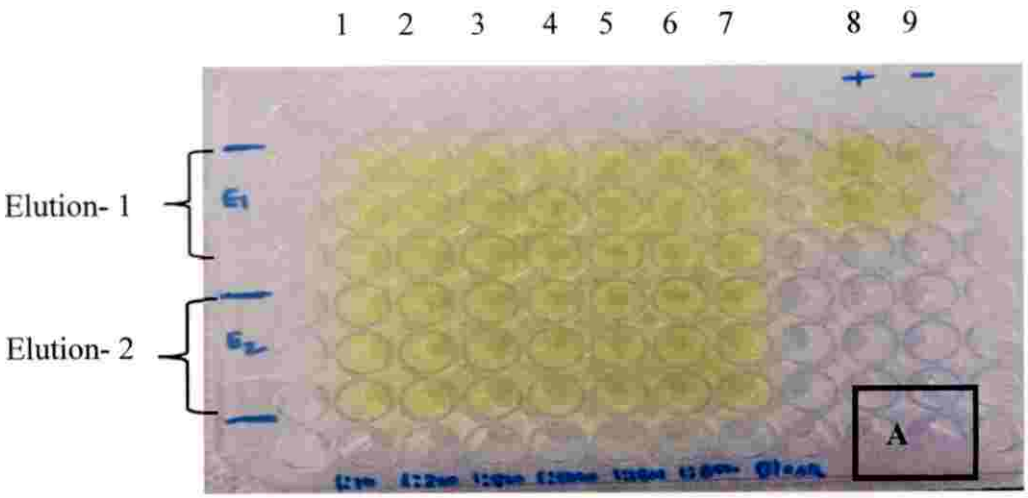
Dilutions	**1: 10**	**1: 200**	**1:500**	**1: 1000**	**1: 1500**	**1: 2000**	**Blank**	**Positive Sample**	**Negative Sample**
Elution- 3	0.285	0.216	0.226	0.214	0.225	0.241	0.206	0.412	0.154
Elution- 2	0.428	0.248	0.251	0.216	0.208	0.224	0.186	+	0.072

4.18. WESTERN BLOTTING USING CMV SPECIFIC ANTISERUM

The eluted protein fraction was separated according to their molecular weight in SDS- PAGE and immunoblotted the protein to nitrocellulose membrane as mentioned in 3.18. Polyclonal CMV- CP specific antibody reacted with expressed coat protein of CMV and a band was visualised at 25 kDa (Plate 12B).

4.19. WESTERN BLOTTING USING ANTI-HISTIDINE MONOCLONAL ANTISERUM

The CMV- CP was immunoblotted to NC membrane by the procedure mentioned in 3.19. Monoclonal anti- histidine antibody didn't react with expressed coat protein of CMV and no band was visualised at 25 kDa.



1- 1: 10, 2- 1: 200, 3- 1: 500, 4- 1:1000, 5- 1: 1500,6- 1: 2000, 7- Blank, 8- + Ve, 9- -Ve

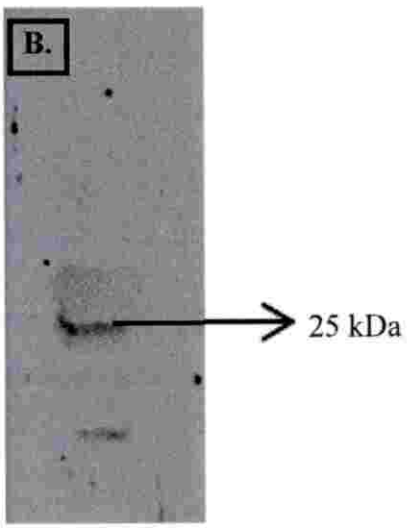


Plate 12: Evaluation of CMV coat protein expressed by recombinant pRSET–C vector, A– Evaluation of CMV coat protein using DAC– ELISA, B- Evaluation of CMV coat protein using western blot

4.21. SUCROSE DENSITY GRADIENT CENTRIFUGATION OF CMV- COAT PROTEIN

An attempted was made to purify the CMV coat protein by sucrose density gradient centrifugation using Tris- NaCl buffer (pH 8.0) and SAT buffer (pH 5.5).

4.21.1. Sucrose density gradient using Tris- NaCl (pH 8.0)

The CMV- CP was purified by 10- 40 per cent sucrose density gradient ultracentrifugation as described in 3.21. Total 18 fractions were collected at the rate of 1.5 ml and electrophoresed by SDS- PAGE. The protein profile indicated that recombinant CP was more in 10- 20 per cent of sucrose gradient. Fractions from 1- 9 were pooled and ultra- pelleted (Plate 13C) and used in SDS- PAGE. Along with protein of interest of 25 kDa, bacterial proteins were also observed (Plate 13A).

4.21.2. Sucrose density gradient using SAT (pH 5.5) buffer

The CMV- CP was purified using SAT buffer of pH of 5.5., as described in 3.21.1. The protein profile in SDS- PAGE showed that protein was more in the pellet, which indicated that CMV coat protein is insoluble in SAT buffer (Plate 13B).

4.22. PURIFICATION OF CMV- CP USING COMMERCIALLY AVAILABLE KIT

E. coli BL21(DE3)pLysS/ pET28a/CMV- CP cells were induced with 0.3 mM IPTG and the protein was purified using tractor buffer and Ni- NTA column as menstioned in section 3.22.

In SDS- PAGE of cell pellet, no protein was observed at 25 kDa. Supernatant, flow through and wash were run in SDS- PAGE and protein band at 25 kDa was observed along with other bacterial proteins. Only 25 kDa protein was visible in all the eluted fractions (Plate 14A).

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4.22.1. Evaluation of CMV- CP produced through His60Gravity column purification kit with commercially available polyclonal antiserum

Different dilutions of coat protein *viz.*, 1: 10, 1: 100, 1: 200, 1: 1000 and blank were used. 1: 100 dilution and flow through possessed maximum absorbance at 405 nm and the same had double the value of negative control. Along with CMV- CP dilutions, positive and negative leaf samples collected from field were also kept. Positive sample had double the value of absorbance than negative sample. Both blank and the negative control revealed almost similar absorbance at 405 nm (Plate 14B). ELISA reading has tabulated in Table 4.6

Table 4.6. Absorbance at 405 nm of protein purified through commercially available kit in DAC-ELISA

Dilution	**1: 10**	**1: 100**	**1: 200**	**1: 1000**	**Blank**	**+ve Sample**	**-ve Sample**
Elution- 2	0.285	0.245	0.179	0.196	0.151	0.464	0.154
Flow through	0.150	0.534	0.189	0.136	0.142	0.396	0.209

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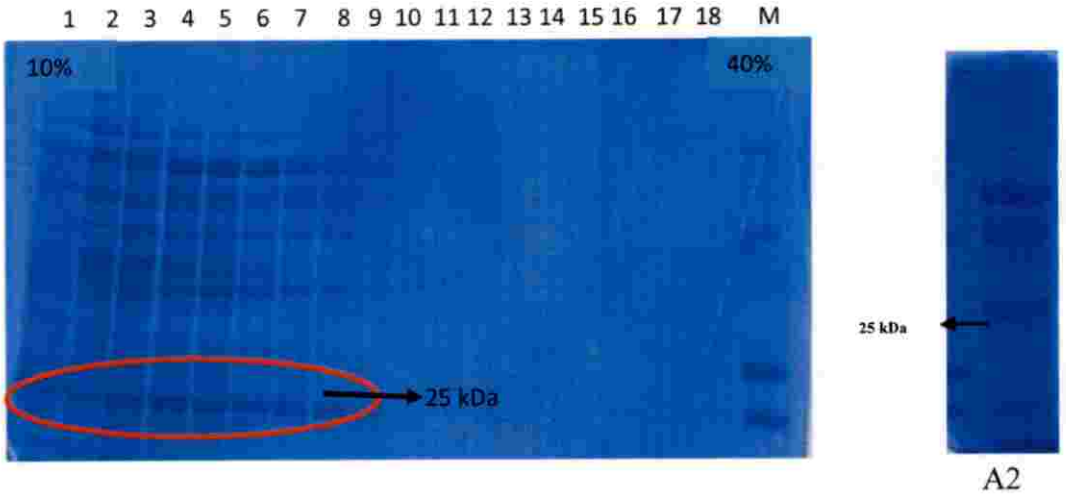
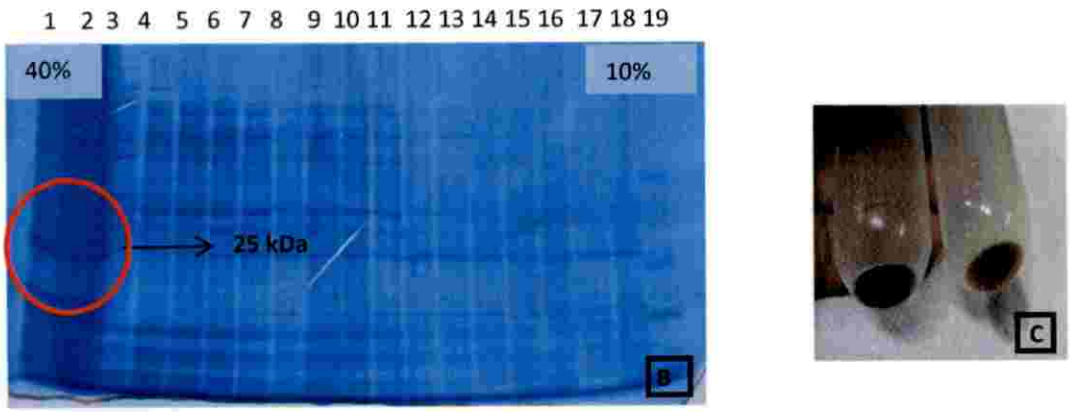


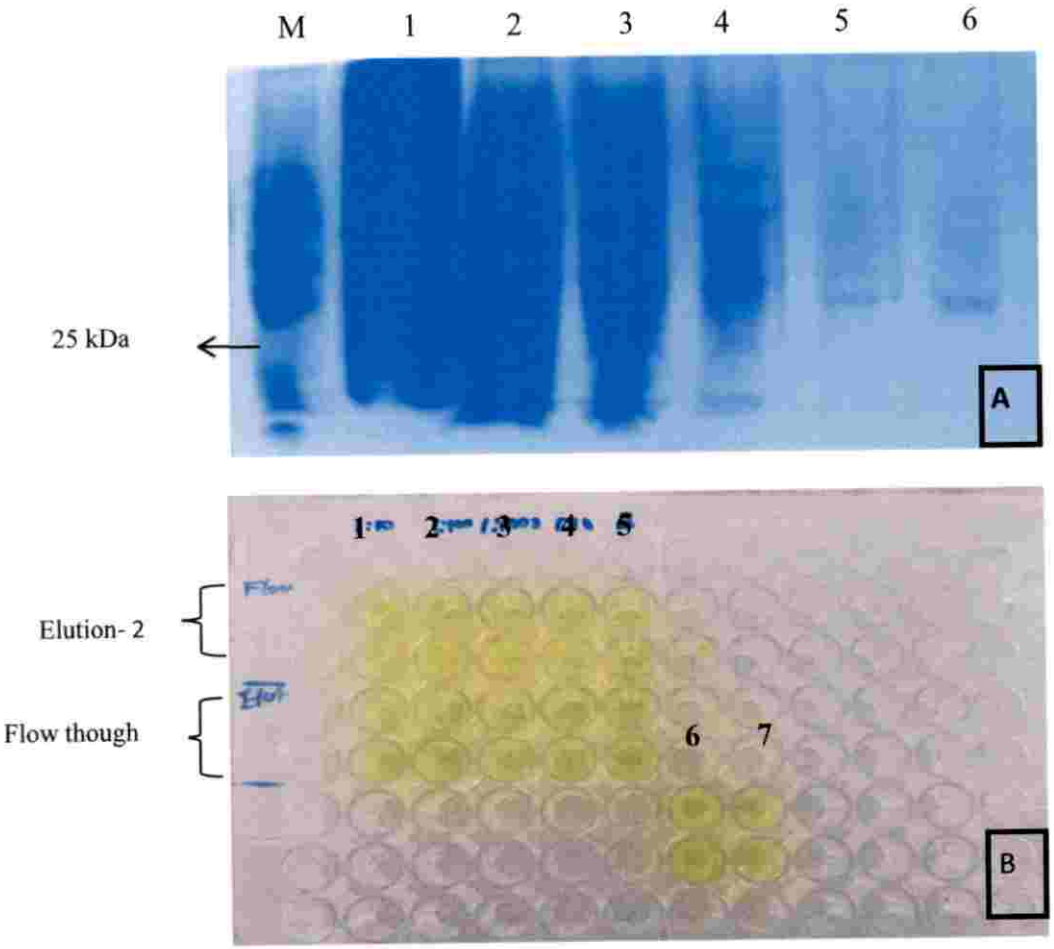
A2

A. Protein profile of sucrose density gradient purified CMV- CP. **A1**- Dissolved in Tris- NaCl (pH 8.0) buffer. M- Protein marker; **A2**– Ultra– pelleted and pooled fraction from 1- 9



B- Protein profile of sucrose density gradient purified CMV- CP, A- Dissolved in SAT buffer (pH 5.5) buffer, Lane 1 and 2: Cell pellet, Lane 3- 19: Sucrose density gradient fractions, **C**- Ultra- pellet of CMV- CP

Plate 13: Sucrose density gradient purification of CMV coat protein



B– DAC– ELISA of CMV coat protein

A- SDS- PAGE gel profile of Ni- NTA purified CMV coat protein. Lane M- Marker. Lane 1- Pellet; Lane 2- Supernatant; Lane 3- flow though, Lane 4- wash, Lane 5 - Elution- 1, Lane 6- Elution- 2

B– Evaluation of CMV coat protein using commercially available antiserum in DAC- ELISA, 1- 1: 10, 2- 1: 100, 3- 1: 200, 4- 1: 1000, 5- blank, 6- + Ve, 7- -Ve

Plate 14. Evaluation of CMV coat protein expressed by recombinant pET28a vector

Discussion

5. DISCUSSION

Banana (*Musa* spp.) is considered as one of the most important fruit crops in the world. It belongs to the genus *Musa* and family *Musaceae* of the order Zingiberales. Almost twenty different viruses are known to cause infection in banana, among which economically important viruses are *Cucumber mosaic virus*, *Banana bunchy top virus*, *Banana streak virus*, *Banana bract mosaic virus* and *Banana mild mosaic virus* (Tripathi *et al.*, 2016).

Effective control of viral diseases depends on the availability of reliable virus detection methods and obligation of the risk factor that each of these diseases poses. According to Niblett *et al.* (1994), the mosaic disease of banana is a nuisance to growers establishing new plantings. The disease is becoming more common in banana orchards, where tissue culture derived planting materials are used.

Different strains of the virus are known to occur. Among all the strains of *Cucumber mosaic virus* infecting banana, heart rot strain causes significant losses, due to rotting of inner leaves leading to death of the plant (Lockhart, 2000). So far, there is no report of heart rot strain from Kerala (Mujtaba, 2017).

Cucumber mosaic virus is transmitted through more than 60 species of aphids, including *Aphis gossypii*, *Rhopalosiphum maidis*, *R. prunifoliae* and *Myzus persicae.* Different methods are available for diagnosing CMV infection in banana *viz.*, symptomatology, indicator plant test, serology, nucleic acid hybridisation and PCR amplification. Among these, serological methods are widely used to detect *Cucumber mosaic virus* from the field (Lockhart, 2000).

Infection due to CMV was observed in widely cultivated banana varieties in Kerala, such as Nendran, Palyankodan, Karpooravally and Rasthali. Yellowish-green bands and patches or mottling can be seen on leaf lamina of young leaves. Diamond shaped chlorotic streaks may also develop on the younger leaves giving

a striped appearance. Subsequently, the leaf become small, often with mosaic mottling and blotches of necrotic tissues and distorted margins. In severe cases, rotted areas are found throughout the leaf sheath and pseudostem. Diseased plants do not reach maturity. They seldom produce bunch of fruit which never matures and is unfit for consumption.

The present study was carried out with the objective to produce recombinant coat protein of CMV infecting banana, which is to be used for antiserum production.

The outline of present study is,

- Development of molecular clones of coat protein gene from *Cucumber mosaic virus* infecting banana
- Production of recombinant virus coat protein through expression vector
- Confirmation and evaluation of recombinant protein with commercially available polyclonal antiserum

The test samples for *Cucumber mosaic virus* were collected based on the external symptoms on leaves from field. Narrow and smaller leaves, dwarf stature of plants were the symptoms observed in net house. The rosette appearance of leaf and obvious inter-venial chlorosis were noticed as characteristic symptoms developed by *Cucumber mosaic virus* isolate NDRNS- 4. Masking of symptoms was also observed. In field, the parallel chlorotic streaks were noticed on infected young leaves, later the leaves became distorted with necrotic tissues and irregular wavy leaf margin. Mosaic pattern on foliage, and occasional leaf deformation, especially in young suckers developing from infected plants were also noticed. Sivaprasad *et al.* (2016) and Mujtaba (2017) also reported similar symptoms for CMV infection in banana. The symptoms of infectious chlorosis of banana sometimes mimic the calcium and boron deficiency in field and vice versa. Heart rot symptoms caused by *Cucumber mosaic virus* were not observed in samples collected during the present study.

In order to confirm the CMV infection in collected samples, DAC- ELISA was carried out as in section 3.2. Each sample was tested by DAC-ELISA using anti- CMV- CP antibody (NRCB Trichy, 1: 2000). In ELISA, generally test samples were considered as positive, when the mean absorbance value of replicated test samples are double than that of healthy samples. Out of eight samples, only NDRNS- 4 showed positive result in ELISA.

Direct antigen coating enzyme linked immune-sorbent assay directly depends on the concentration of viral particle in samples. Hence, ELISA can be considered as a semi-quantitative test to estimate viral load in the given sample. Compared with other serological methods used to detect CMV from field, ELISA is having higher sensitivity (Mujtaba, 2017).

Sero-diagnosis of symptomatologically selected samples using CMV- CP specific polyclonal antiserum revealed the presence of *Cucumber mosaic virus* (Family: *Bromoviridae*, Genus: *Cucumovirus*) in the test samples. For molecular cloning purposes, sample with higher CMV concentration *viz.,* NDRNS- 4, KANC- 2 and KANC- 4 were selected.

Since CMV is an RNA virus, the first step of molecular cloning is isolation of total RNA from infected samples. Nucleic acid based detection methods generally depend on the purity of RNA isolated. As per Ghangal *et al.* (2009), young to middle aged leaves are suitable for isolation of high quality RNA, since they contain less amount of tannin and phenol. Hence, for the present study, total RNA was isolated from young to middle aged leaves using RNeasy plant mini kit and observed in 1.2 per cent agarose gel. Two bands (*i.e.*, 28S and 18S) of RNA were observed without DNA and protein contamination, proving good quality of RNA and hence proceeded to further studies.

Complementary DNA (cDNA) was synthesised from total RNA isolated by RevertAid H minus first strand cDNA kit. Reverse transcriptase polymerase chain reaction was employed for the detection of CMV using synthesised cDNA from diseased samples. Amplicon at 750 bp was obtained as reported by Hu *et al.* (1995); Cherian *et al.* (2004) and Khan *et al.* (2012). No amplification was obtained at 750 bp, for healthy and diluted leaf samples.

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Low virus titre of infected plant samples were the major problem of traditional virus detection methods *viz.*, electron microscopy, host plant assay and serological technique. *Cucumber mosaic virus* coat protein specific primer based technique is capable enough to amplify the cDNA templates (Kumar *et al.*, 2005).

Cherian *et al.* (2004) and Mujtaba (2017) amplified CMV- CP at 750 bp and 700 bp, respectively from Kerala using CMV-CP specific primer.

The CMV- CP amplicon was purified and ligated to pGEM- T vector using T- A cloning principle. The recombinant plasmid was further transformed into *E. coli* DH5α cells. Positive clones were selected based on blue-white screening, a technique used for the detection of successful ligations in vector-based gene cloning (Plate 3A).

While vector and gene of interest were subjected to ligate, three forms of plasmids could be formed *viz.*, self-ligated vectors, positive clone and wrong recombinant plasmids. Alpha-complementation is the principle behind the blue-white screening. The lac-Z gene product (β- galactosidase) is a tetramer and each monomer is made of two parts *i.e.*, lacZ- alpha and lacZ- omega. Due to insertion of gene, the alpha fragment was deleted which resulted the non- functioning of the omega fragment. Non- recombinant self-ligated plasmid was also successfully transformed into *E. coli* DH5α cells, they would express functional β-galactosidase. Recombinant colonies express non-functional β -galactosidase. β-galactosidase is responsible for breaking of X-gal (5- bromo- 4- chloro- 3-indolyl- β- D-galactopyranoside) to 5- bromo- 4- chloro- Indoxyl which appeared as blue in colour. When the recombinants are allowed to grow in medium containing X-gal and IPTG (isopropyl thio galactoside), the non-recombinant colonies will appear blue, whereas recombinants white.

For the confirmation of CMV- CP gene in the recombinant white colonies, PCR amplification using CMV-CP specific primers followed by restriction digestion and sequencing were carried out. Cherian *et al.* (2004) and Mujtaba (2017) had cloned CMV- CP gene of *Cucumber mosaic virus* infecting banana to pGEM- T cloning vector and confirmed through colony PCR. Restriction

digestion of the recombinant plasmid followed by sequencing was reported by Sabharwal (2017) for the confirmation of PVBV- CP recombinants in pRSET- C vector.

The primer was designed in order to amplify CMV- CP gene along with restriction sites selected from expression vector. Since pRSET- C and pET28a were used for expression studies, *Bam*H1 and *Nhe*1 sites were preferred as these sites will not interfere with the sequence of CMV- CP and are present on the either sides of multiple cloning sites. Restriction sites were added to both forward and reverse primers. Linker sequences were added on the 5' ends of both forward and reverse primers, for proper restriction digestion. Triplets of guanidine and cytosine was added as linker sequences in both the primers to tally the total GC content as well as conserve the overall protein translation frame. Start codon and stop codon were added accordingly

- Primer was designed according to the flowchart given below,

5' 3'

Linker	Start or stop codon	Restriction sites	CMV-CP gene

Based on the sequences available in the NCBI data base, Pandey (2015) designed primer complementary to CMV- CP. Recognition sites responsible for both *Bam*H1 and *Eco*R1 were added to both forward and reverse primer respectively. Using the designed primer, CMV- CP gene of 750 bp was ligated to pRSET- B vector.

Hema *et al.* (2003) designed primer for expression analysis of *Sugarcane streak mosaic virus* coat protein gene. The SCSMV- CP specific primer was designed along with recognition sites of *Eco*R1 and *Hind*III on forward and reverse primer, respectively.

The annealing temperature of designed primer was standardised through gradient PCR as 55°C. The CMV- CP gene was amplified using designed primer and *Pfu* DNA polymerase enzyme and the amplicon was obtained at 750 bp.

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Pfu DNA polymerase is a hyper thermophilic enzyme isolated from *Pyrococcus furiosus*. The enzyme is used to amplify DNA in PCR. Compared to *Taq* DNA polymerase, *Pfu* DNA polymerase has superior thermostability and 3' to 5' exonuclease proofreading properties (Verkuil *et al.*, 2008). Consequently, *Pfu* DNA polymerase generated PCR amplicons will have fewer errors than *Taq* generated PCR fragments. *Pfu* DNA polymerase in PCR reactions results in blunt ended PCR products.

Lundberg *et al.* (1991) and Vercuil *et al.* (2008) amplified DNA fragments using high fidelity polymerase enzyme. Gulati *et al.* (2016) and Sabharwal (2017) standardaised the annealing temperature of designed primer and amplified *Tobacco streak mosaic virus* and *Pepper vein banding virus* coat protein gene using *Pfu* DNA polymerase. Mujtaba (2017) also standardaised the annealing temperature of CMV- CP specific designed primer and amplified the gene of interest using *Taq* DNA polymerase.

The expression vectors and the amplicon were digested using selected restriction enzymes and later ligation was carried out in the presence of T4 DNA ligase enzyme. Since the gene responsible for blue-white screening is absent in the selected expression vectors, positive clones were selected according to the molecular weight of recombinant plasmid observed in 0.8 per cent agarose gel. Later, the clones were confirmed by PCR amplification using *Taq* DNA polymerase and CMV- CP specific primers.

Hema *et al.* (2003) reported the expression of *Sugarcane streak mosaic virus* coat protein in pRSET- C vector. Pandey (2015) expressed coat protein gene of CMV infecting cucumber, using pRSET- B vector. Kim *et al.* (2016) cloned CMV- CP into pET21a vector and expressed 25 kDa protein. Gulati *et al.* (2016) and Sabharwal (2017) expressed *Tobacco streak mosaic virus* coat protein and *Pepper vein banding virus* encoded protein in pRSET- C vector.

Expression in *E.coli* is fast, climbable and has minimum post-translational modifications. Such expressed protein is highly suitable for structural and

crystallographic studies (Hartley, 2006). In the present study, two *E. coli* strains were selected as expression host, *E. coli* BL21(DE3)pLysS and *E. coli* Rosetta(DE3)pLysS.

E. coli BL21(DE3)pLysS and *E. coli* Rosetta(DE3)pLysS strains are the most widely used hosts for protein expression from recombinant plasmids and have the advantage of being deficient in both *lon* and *ompT* proteases. DE3 indicates that the host is a lysogen of λDE3, and therefore carries a chromosomal copy of the T7 RNA polymerase gene under control of the lacUV- 5 promoter. Such strains are suitable for the production of protein from target genes cloned in expression vectors by induction with IPTG. pLysS strains express T7 lysozyme, which further suppresses basal expression of T7 RNA polymerase prior to induction, thus stabilising recombinant plasmids encoding lethal proteins.

Hema *et al.* (2003) expressed recombinant *Sugarcane streak mosaic virus* coat protein gene in *E. coli* BL21 (DE3)pLysS/pRSET- C/ CMV- CP system. Khan *et al.* (2012) cloned *Cucumber mosaic virus* coat protein gene to pQE3 cloning vector and expressed virus specific coat protein under the control of IPTG inducible T_7 lac promoter. Koolivand *et al.* (2017) expressed *Cucumber mosaic virus* coat protein in *E. coli* Rosetta(DE3)pLysS cells.

The CMV- CP gene was induced by IPTG, which is an inducer molecule for the Lac and pET or pRSET expression systems. Isopropyl β-d-1-thiogalactopyranoside binds to the Lac repressor, whose natural ligand is the disaccharide lactose, resulting in repression of DNA binding. It is used in recombinant protein production as, unlike lactose, it is non- metabolisable and therefore, is not degraded by bacteria over time. The T7 promoter of phage genome controls the recombinant plasmid in expression host. T7 RNA polymerase is required for translation of gene of interest, which was encoded by host cell genome known as the DE3 locus.

The expression of T7 RNA polymerase was regulated by lacUV5 promoter. To express the recombinant gene, IPTG was added to the system, which

is a ligand for the Lac repressor. When combine with IPTG, Lac repressor removes the lacUV5 promoter, enabling *E. coli* RNAP to transcribe the T7 gene-1, encoding the T7 RNAP. T7 RNAP is then able to activate the promoter on the expression vector and transcribe the recombinant CMV- CP gene. In the present study, CMV- CP gene was induced with 0. 3 mM IPTG for six hours, at 30°C.

El-Borollosy and Hassan (2014) expressed *Cucumber mosaic virus* coat protein through the induction of 100 µM IPTG. Sabharwal (2017) induced *E. coli* BL21(DE3)pLysS/pRSET- C/PVBV- CP system using 0.3 mM IPTG and expressed the virus encoded protein.

Solubility of protein is an important pre-requisite for structural studies. Obtaining sufficiently higher concentrations of protein in soluble fraction remains a major experimental challenge. To get a suitable buffer for solubilisation of the expressed protein is a trial and error process with relatively low success rate (Chow *et al.*, 2006).

The pH should be at least one unit away from the theoretical isoelectric point (pI) to keep the protein charged so that the electrostatic repulsion keeps the protein away from aggregating. The theoretical pI can be estimated from ExPASy- Translate Tool using deduced amino acid sequences with maximum ORF. The CMV- CP sequence obtained as part of this study was deduced as mentioned above and observed the theoretical pI as 10. 39 and hence pH of the buffer was estimated as 8.0. Tris- Nacl of pH 8.0 was selected as suitable buffer for protein purification. Gulati *et al.* (2016) solubilised the coat protein of *Tobacco streak mosaic virus* in Tris- NaCl of pH 8.5.

The coat protein of CMV is produced endogenously inside the bacterial expression system. Sonication apply high-frequency ultrasound energy (generally >20 kHz) to the samples to agitate and disrupt the cell membranes, ultimately causes cell lysis. Heat energy will be generated during the course of sonication, so that the sample should be placed in ice. Plastic beaker was used for sonication to avoid unwanted damages.

Since the 6X histidine tagged recombinant protein present along with bacterial protein, selective purification of the protein was carried out using Ni-NTA agarose is a nickel- charged affinity resin. Poly histidine tag is a thread of amino acid histidine residue on either N or C terminal of expressed protein. Ni-NTA purification mainly depends upon the 6X histidine tag. The tag possesses high affinity towards the Ni^{2+} ion and thus purification was carried out.

Imidazole is one of the major components of buffers used in metal affinity column chromatography, it competes with histidine tag to bind with metal charged resin, hence, for wash buffer low concentration of imidazole was added whereas for elution buffer higher concentration of imidazole is used to displace the 6X his-tagged protein from metal affinity column during purification.

Concentration of protein in each elution fraction was estimated using Bradford reagent. Blue colour was observed up to three elutions and later the solution remained as brown, indicated the absence of protein. It is a colorimetric protein assay based on absorbance of dye Coomassie Brilliant Blue G-250. Under acidic conditions, the brown coloured dye will be converted to blue, when binds to protein.

Protein expressed through pRSET- C vector was purified using Ni- NTA column chromatography and was electrophoresed in SDS- PAGE. The absence of 25 kDa protein in cell pellet indicates the high solubility of coat protein in Tris-NaCl buffer (pH 8.0). Each elution was quantified in nano drop and maximum quantity was observed in elution- 2. Similar result was observed by Khan *et al.* (2012).

The protein was also expressed in pET28a vector and purified through Ni-NTA column chromatography using commercially available kit, 25 kDa protein corresponding to CMV coat protein was visualised in both flow through and wash fraction along with elutions. The presence of protein in the flow through and wash fractions may be due to improper binding of recombinant protein with Ni-NTA beads or less amount of imidazole in elution buffer.

Hochuli *et al.* (1987) purified histidine tagged protein using metal affinity ligand based column. Khan *et al.*(2012), Pandey (2015) and Gulati *et al.* (2016) purified the recombinant coat protein of *Cucumber mosaic virus* and *Tobacco streak mosaic virus*, respectively by the same technique.

The recombinant protein was serologically evaluated by western blotting and DAC- ELISA, using polyclonal anti-CMV- CP antibody and monoclonal anti-histidine antibody.

Western blotting enables the electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose membrane. Animal derived polyclonal primary antibody was added to the NC membrane and it would be later detected by enzyme conjugated secondary antibody. The secondary antibody was visualised through staining, allowing indirect detection of the protein of interest. Protein from SDS- PAGE was electro- blotted to NC membrane using an electric current at 80 mA, to pull the negatively charged proteins from the gel towards the positively charged anode, and into the NC membrane. The protein moved from the gel to the membrane by maintaining the native structure.

Transferred CMV coat protein from SDS- PAGE gel in to nitro- cellulose membrane and the immobilised proteins were detectable by immunological procedures. The immobilisation was carried out in the presence of transfer buffer. The role of methanol in transfer buffer is to help good transfer of small proteins to membrane. Methanol removes the SDS from the proteins, which eliminates the charge of the proteins and causes the gel to shrink. Later, the protein of interest was estimated by antibody- antigen interaction. Both qualitative as well as quantitative means of protein estimation is enabled (Towbin *et al.*, 1979).

Detection of CMV- CP by western blotting using polyclonal anti-CMV-CP antibody was reported by Khan *et al* (2012) and Rostami *et al.* (2014).

Western blotting was carried out using monoclonal anti-histidine antibody, in which no band was visualised corresponding to 25 kDa. It may be due to the

fact that of CMV coat protein regains the native structure of spherical capsid protein during electro- blotting. The poly histidine tag, the paratope of monoclonal anti-histidine antibody may be oriented towards the centre of spherical CMV- CP and thus masked the epitope-paratope interaction, later resulted in no bands at 25 kDa.

The expressed virus coat protein was evaluated against commercially available antiserum in DAC-ELISA. The 1: 10 dilution of pRSET- C/ CMV- CP recombinant protein and 1: 100 dilution of pET28a/CMV- CP recombinant protein produced maximum absorbance at 405 nm.

The expressed protein was purified through sucrose density gradient centrifugation. Ultra-centrifugation at high speed separates small particles such as viruses and proteins from the crude lysate. Sucrose density gradient ultra-centrifugation is a powerful technique for fractionating proteins according to their density.

Supernatant of *E. coli* BL21(DE3)pLysS cell was collected and ultra-centrifuged. The ultra- pellet obtained was further dispensed in Tris-NaCl buffer (pH 8.0) and later the crude lysate of protein was layered on the surface of a sucrose density gradient ranged from 10 to 40 per cent whose density was observed to increases linearly from top to bottom. Electrophoresis of samples collected from sucrose density gradient showed more concentration of protein in 10- 20 per cent. Along with CMV coat protein, bacterial proteins were also observed.

The ultra- pellet obtained after centrifugation at 28,000 rpm was later dispensed in SAT buffer (pH 5.5) in order to check the solubility of CMV coat protein in acidic buffer. Along with fractions collected from sucrose density gradient, cell pellet collected during harvesting of *E. coli* BL21(DE3)pLysS cells after sonication were also observed in SDS- PAGE. More concentration of 25 kDa protein was observed on cell pellet, than density gradient fractions, which means that the buffer is not suitable for solubilising CMV coat protein.

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Gulati *et al* (2016) and Sabharwal (2017) purified the recombinant virus encoded protein of *Tobacco streak mosaic virus* and *Pepper vein banding virus*, respectively using sucrose density gradient.

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Summary

6. SUMMARY

The study entitled "Development of recombinant coat protein for immunodetection of *Cucumber mosaic virus* infecting banana" was carried out using existing facilities of Department of Biochemistry, Indian Institute of Science, Bangalore, Division of plant Pathology, Banana Research Station, Kannara, Department of Plant Pathology, CoH, Vellanikkara, Department of Veterinary Microbiology, CoVAS, Mannuthy and Department of Biochemistry, Amala Cancer Research Centre, Thrissur during 2018- 2019. Present study was carried out to produce recombinant coat protein, later, can be used for production of high quality antiserum for the detection of *Cucumber mosaic virus* infecting banana.

Banana is infected by four well characterised plant viruses *viz*., *Banana bunchytop virus*, *Cucumber mosaic virus*, *Banana bract mosaic virus* and *Banana streak virus*. Among these viruses, CMV causes devastating effect on tissue culture banana plants. Various symptoms of CMV were observed under natural conditions like, diamond shaped discontinuous lesions, severe mosaic with extreme distortion and reduction of leaf lamina.

Direct antigen coating immune sorbent-assay was carried out for immuno-diagnosis of *Cucumber mosaic virus* samples collected from the field. Based on the readings, KANC- 2, KANC- 4 and NDRNS- 4 were selected for molecular detection of the CMV coat protein. In order to develop molecular clones of CMV coat protein, the total RNA from the selected leaf samples were isolated and cDNA synthesised. Reverse transcriptase polymerase chain reaction was carried out using CMV- CP specific primer, reported by Cherian *et al*. (2004) and CMV-CP amplicon was generated at 750 bp in 1.2 per cent agarose gel. KANC- 2 and NDRNS- 4 were shown positive amplification whereas no amplification was observed in KANC- 4 at 750 bp.

The PCR product was purified and ligated to pGEM- T linear plasmid vector, later transformed the recombinant plasmid (i.e., pGEM- T/ CMV- CP) to

E. coli DH5α cells. Positive clones were selected according to blue- white screening, in which white colonies were considered as recombinants and blue ones as non-recombinants. Individually selected each white colony and subcultured and later confirmed the positive ligation through colony PCR using CMV- CP specific primer. The recombinant plasmid was isolated by alkali- lysis method and digested with restriction enzyme, *Eco*R1 which released 750 bp CMV- CP fragment from recombinant pGEM- T/CMV- CP plasmid. Then the recombinant plasmid was sent for sequencing, thus confirmed *E. coli* DH5α/pGEM- T/ CMV- CP cloning.

Coat protein specific reverse and forward primers were designed for expression analysis of CMV- CP gene. The primer was designed using CMV- CP specific sequence along with sequences responsible for restriction enzymes *Bam*H1 and *Nhe*1. Using the designed primer, CMV- CP gene was amplified using high fidelity *Pfu* DNA polymerase enzyme. The resulted amplicon contained recognition sites of both restriction enzymes *Bam*H1 and *Nhe*1. Both expression vector as well as the amplicon was subjected for ligation using T4 DNA ligase at 16°C temperature. The recombinant plasmids (pRSET- C/ CMV- CP or pET28a/CMV- CP) were transformed to *E. coli* DH5α cells. Later isolated the plasmids and selected the positive clones by observing the increase in molecular weight of recombinant plasmids compared to normal pRSET- C or pET28a plasmids. Each selected plasmid were subjected to PCR using CMV- CP specific primer to confirm the ligation. The expression clones were sent for sequencing and hence confirmed the recombination.

The recombinant expression plasmids were transformed to *E.coli* BL21(DE3) pLysS and standardized the expression of CMV- CP. Using ExPASy- protein translation tool, isoelectric point of CMV coat protein was elucidated as 10.3 and pH of buffer suitable for solubilize the recombinant coat protein was estimated as 8.0. As per Gulati *et al.* (2016), Tris- NaCl buffer of pH 8.0 was selected to solubilize the recombinant protein. The recombinant fusion protein was further purified through Nitrilotriacetic acid column purification, in which

agarose coated Nickel beads were used for binding the 6X histidine tagged protein. Low and high concentrations of imidazole were added to both wash and elution buffer, respectively. Each fractions *viz.*, cell pellet, supernatant, flow through, wash and elution was collected, later observed these fractions in SDS-PAGE. No protein at 25 kDa was observed in cell pellet, implies that the recombinant coat protein is completely soluble in Tris- NaCl buffer (pH 8.0).

The expressed CMV- CP was confirmed through DAC- ELISA and western blotting. Commercially available antiserum of titre 1: 2000 was used in DAC ELISA against different dilutions of antigen *i.e.*, CMV- CP. 1: 10 dilution of 2^{nd} elution possessed maximum absorbance at 405 nm in an ELISA reader. A band corresponds to 25 kDa was noticed on nitrocellulose membrane during Western blotting, confirmed the expression of *Cucumber mosaic virus* recombinant coat protein.

The recombinant coat protein was further attempted to purify through sucrose density gradient. *E. coli* BL21(DE3)pLysS/ pRSET- C/CMV- CP cells were harvested and the supernatant was ultra- pelleted. Dissolved the ultra-pellet in both Tris- NaCl (pH 8.0) and SAT buffer (pH 5.5). Density gradient of 10- 40 per cent sucrose dissolved in suitable buffer was prepared and kept at 4°C, overnight. The ultra- pellet dissolved in Tris- NaCl buffer was loaded in the density gradient and later partially purified. Each fraction was collected in 1.5 ml micro centrifuge tube and observed the fractions in SDS- PAGE. The CMV- CP was concentrated in 10- 20 per cent of density gradient. Apart from virus coat protein, contaminant bacterial protein was also observed in the same fraction of sucrose density gradient.

Coat protein of CMV ultra- pellet was dissolved in SAT buffer of pH 5.5, carried out the sucrose density gradient centrifugation and later observed the density gradient fractions in SDS- PAGE. More protein was observed in cell pellet fraction, which indicates that the protein was not soluble in pH 5.5 *i.e.*, acidic pH.

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Cucumber mosaic virus coat protein was attempted to purify through commercially available His60 Ni Gravity Columns. *E. coli* BL21(DE3)pLysS/pET28a/CMV- CP cells were harvested and collected the supernatant. The supernatant was allowed to bind with Ni^{2+} agarose beads and later purified through Ni- NTA column chromatography. Each fractions like, cell pellet, supernatant, flow through, wash and elution were observed in SDS- PAGE. No protein band was visualized at 25 kDa in cell pellet. More protein at 25 kDa was observed in flow through than elution, which nessacitates further validation of purification process. The protein fraction of flow through and elution were tested using commercially available antiserum of 1: 2000 dilution in DAC-ELISA. Among the various dilutions of CMV- CP and observed more absorbance in 1: 100 dilution of flow through than dilutions of elution.

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Development of recombinant coat protein for immunodetection of *Cucumber mosaic virus* infecting banana

By

Alan C. Antony

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Abstract of the thesis

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Kerala Agricultural University, Thrissur

Department of Plant Pathology

COLLEGE OF HORTICULTURE

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Abstract

Banana (*Musa* spp.) is infected by four well characterised plant viruses *viz*., *Banana bunchy top virus*, *Cucumber mosaic virus* (CMV), *Banana bract mosaic virus* and *Banana streak virus*. Among these, CMV causes devastating effect on tissue culture banana plants. The study entitled "Development of recombinant coat protein for immunodetection of *Cucumber mosaic virus* infecting banana" was carried out using existing facilities of Department of Biochemistry, Indian Institute of Science, Bangalore, Division of Plant Pathology, Banana Research Station, Kannara and Department of Plant Pathology, College of Horticulture, Vellanikkara, Thrissur during 2018- 2019. The present study was carried out to produce recombinant coat protein, which can be utilised later for producing high quality antiserum for the detection of CMV infecting banana.

Cucumber mosaic virus infected samples were collected based on various characteristic symptoms and screened by direct antigen coating immunosorbent assay using commercially available CMV polyclonal antiserum. Isolate namely, KANC- 4, KANC- 2 and NDRNS- 4 showed maximum absorbance at 405 nm and hence selected for molecular detection using reverse transcriptase polymerase chain reaction with CMV- CP specific primer. The PCR product was purified and CMV- CP amplicon of NDRNS- 4 isolate was ligated to pGEM- T linear plasmid vector, which was later transformed into *E. coli* DH5α cells. Positive clones were selected according to blue-white screening. Cloning *i.e*., *E.coli* DH5α/pGEM-T/CMV- CP was confirmed through colony PCR using coat protein specific primer, restriction digestion of recombinant plasmids using *Eco*R1 enzyme followed by sequencing.

The vectors *viz*. pRSET- C and pET28a were selected for the expression of CMV- CP gene in *E. coli*. Coat protein specific forward (5'GGG GCT AGC ATG GAC AAA TCT GAA TCA ACC3') and reverse primers(5'CCC GGA TCC TTA CTC TCC ATG GCG TTT AG 3') were designed along with recognition sites of restriction enzymes *Bam*H1 and *Nhe*1.The annealing temperature of designed

primer was standardised as 55°C using gradient PCR. The coat protein gene of CMV was amplified at 750 bp using designed primers and high fidelity *Pfu* DNA polymerase enzyme. Expression vectors as well as amplicon were subjected to ligation and the recombination in expression plasmids (pRSET- C/ CMV- CP and pET28a/CMV- CP) were confirmed through PCR and sequencing. The plasmid with maximum homology *i.e.*, pRSET-C/CMV- CP was selected for further studies.

The recombinant plasmid was transformed into *E. coli* BL21(DE3)pLysS cells for the expression of CMV- CP gene and the expression of 25 kDa recombinant CMV coat protein was confirmed in 12 per cent sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS- PAGE). Tris- NaCl buffer of pH 8.0 was selected for solubilising the recombinant protein using ExPASy - protein translation tool. The recombinant protein was further purified through Nitrilotriacetic acid column purification, in which the 6X histidine tagged recombinant protein was bound with agarose coated nickel beads. Buffers containing imidazole were used for the elution of histidine tagged recombinant protein, since imidazole competes with histidine for the binding site in nickel beads. Each fraction *viz.*, cell pellet, supernatant, flow through, wash and elution were collected and later detected for protein using SDS- PAGE. Absence of 25 kDa protein in cell pellet indicated that the recombinant coat protein completely soluble in Tris- NaCl buffer (pH 8.0).

Confirmation of recombinant coat protein was carried out through DAC-ELISA and western blotting using commercially available polyclonal CMV antiserum (1: 2000; NRCB, Trichy).

The recombinant coat protein developed through this study could be utilised for large scale production of antiserum for immunodetection of CMV.

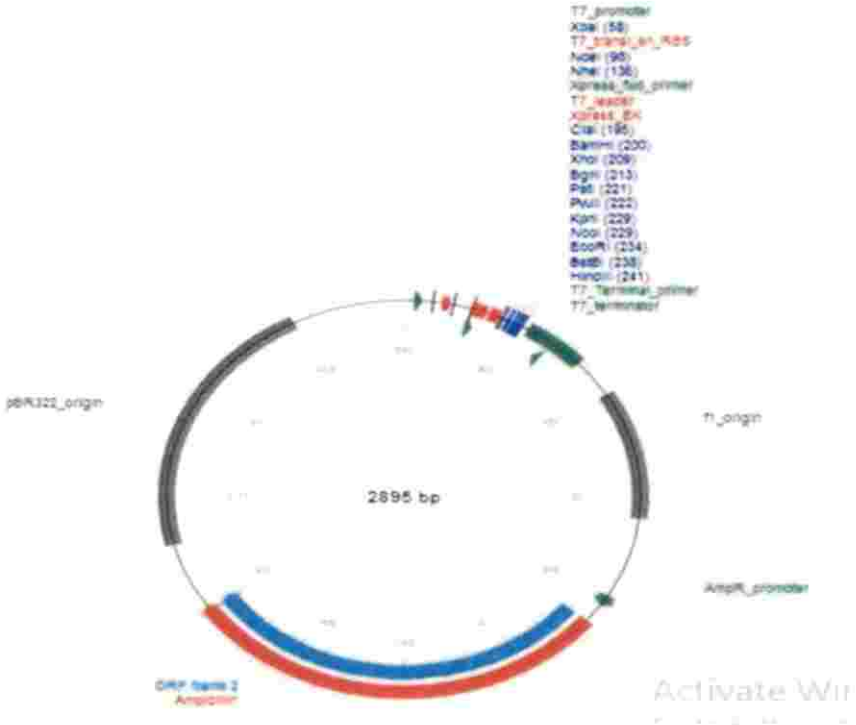
Appendices

APPENDICES

1. Sequence result of CMV- CP gene

>CMV_CP_CLONE
5'CGTTGTAAAACGACGGCCAGTGAATTGTAATACGACTCACTATA
GGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGG
CCGCGGGAATTCGATTCATCGACCATGGACAAATCTGAATCAACC
AGTGCTGGTCGTAACCGTCGACGTCGTCCGCGTCGTGGTTCCCGCT
CCGCTTCCTCCTCCGCGGATGCTACATTTAGAGTCCTGTCGCAACA
GCTTTCGCGACTTAATAAGACGTTAGCAGCTGGTCGTCCTACTATT
AACCACCCAACCTTTGTGGGTAGTGAGCGCTGTAGACCCGGATAC
ACGTTCACATCAATTACCCTGAAGCCACCAAAAATAGACCGAGGG
TCTTATTATGGTAAAAGGTTGTTACTTCCTGATTCAGTTACTGAGTT
CGATAAGAAGCTTGTTTCGCGCATTCAAATTCGAGTTAATCCTTTG
CCGAAATTTGATTCTACCGTGTGGGTGACGGTCCGTAAAGTTCCTG
CCTCCTCGGACCTGTCCGTTGCCGCCATCTCTGCTATGTTTGCGGA
CGGAGCCTCACCGGTACTGGTTTATCAGTACGCCGCATCCGGAGTT
CAAGCTAACAACAAGTTGTTGTATGATCTTTCGGCGATGCGCGCTG
ATATTGGCGACATGAGAAAGTACGCCGTGCTCGTGTATTCAAAAG
GCGATACGCTAGAGACGGATGAGCTAGTACTTCATGTCGACACTG
AGCACCAACGCATTCCCACATCTGGGGTGCTCCCAGTTTGAACTCG
TGTTTTCCAGAACCCTCCCTCCGATTTCTGTGGCGGGAGCTGAGTT
GGCAGTGTTGCTATAAACTGCTGAAGTCACTAAACGCCATGGAGA
GAATCACTAGTGAATTCGCGGCCGCCTGCACGTCGACCATATGGG
AGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGT
CACCTAAATAGCT3'

2. Vector map of pRSET- C vector



3. Vector map of pET28a vector