MOLECULAR CLONING AND CHARACTERISATION OF COAT PROTEIN GENE OF *BANANA BRACT MOSAIC VIRUS*

by

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THESIS

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2016

DECLARATION

I hereby declare that the thesis entitled **"Molecular cloning and characterisation of coat protein gene of *Banana bract mosaic virus"*** is a bonafide record of research work done by me during the course of research and that it has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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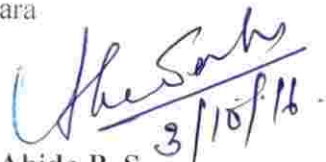College of Horticulture, Vellanikkara

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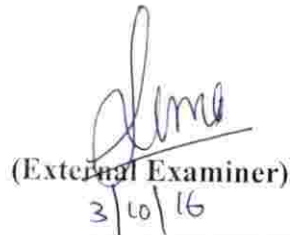College Of Horticulture, Vellanikkara

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ABBREVIATIONS

°C	:	Degree Celsius
BBrMV	:	*Banana bract mosaic virus*
BBrMD	:	Banana bract mosaic disease
BBTV	:	*Banana bunchy top virus*
BLAST	:	Basic Local Alignment Search Tool
BSV	:	*Banana streak virus*
BSA	:	Bovine serum albumin
cM	:	Centimorgan
Cm	:	Centimeter
CMV	:	*Cucumber mosaic virus*
CP	:	Coat protein
M	:	Meter
CPBMB	:	Centre for Plant Biotechnology and Molecular Biology
$CaCl_2$	:	Calcium chloride
cDNA	:	Complementary Deoxyribose Nucleic Acid
dNTP	:	Di-Nucleotide Triphosphate
DNA	:	Deoxyribose Nucleic Acid
DAC-ELISA	:	Direct Antigen Coating Enzyme Linked Immuno Sorbent Assay

DIBA	:	Dot Immuno Binding Assay
FAO	:	Food and Agriculture Organization
G	:	Gram
KAU	:	Kerala Agricultural University
Kb	:	Kilo base
M	:	Molar
$MgCl_2$	:	Magnesium chloride
Mg	:	Milligram
min.	:	Minute
Mm	:	Millimeter
Ml	:	Milliliter
NCBI	:	National Center for Biotechnological Information
Nm	:	Nanometer
Ng	:	Nano gram
µg	:	Microgram
µl	:	Microliter
µm	:	Micromole
OD	:	Optical Density
ORF	:	Open Reading Frame
pH	:	Hydrogen ion concentration

PCR	:	Polymerase Chain Reaction
Ppm	:	Parts per million
PVP	:	Poly vinyl pyrolidone
Rpm	:	Revolution per minute
%	:	Per cent
RAPD	:	Random Amplified Polymorphic DNA
RT-PCR	:	Reverse Transcription PCR
RNA	:	Ribonucleic Acid
RNAse	:	Ribonuclease
s.	:	Second
TAE	:	Tris Acetate EDTA
TBE	:	Tris Borate EDTA
Taq	:	*Thermus aquaticus*
U	:	Unit
UTR		Untranslated Region
V	:	Volt

INTRODUCTION

1. Introduction

Banana (*Musa* spp.), considered as 'tropical treasure' is grown extensively in the tropical and sub tropical regions of the world. The word 'banana' comes from the Arabic word 'banan' which means finger. Generally, the term banana embraces a number of species or hybrids within the *Musa* genus of the family Musaceae (Mortan and Miami, 1987). According to Vavilov (1935), the center of origin of *Musa* is Indo-China and Malayan Archipelago.

Banana and Plantain are the second largest fruit crops of the world cultivated both in the tropical and subtropical regions of the world. The global production of banana is around 102.02 lakh tonnes and total area under banana is around 45.44 lakh hectares (FAO, 2013). India, China, Philippines, Ecuador and Brazil are the major producers of banana in the world (FAO, 2013). Asia-Pacific countries, particularly India, China and Philippines are the major cultivators of banana, contributing more than 45 per cent of the global production. India ranks first in the world, with respect to area and production. Within the country, it is cultivated in all the states. However, major banana growing states are Tamil Nadu, Karnataka, Andhra Pradesh, Maharashtra, Gujarat, Kerala, Assam, West Bengal, Bihar, Madhya Pradesh, Odisha, Chhattisgarh and Uttar Pradesh with an area of 8.8 lakh hectares and production of 30 lakh tonnes in 2014-15. In Kerala, banana is grown in almost all districts with an area of 1.85 lakh hectares and production of 11.6 lakh tonnes during 2014-15 (DAC & FW, 2015).

Banana is also the most remunerative fruit crop of Kerala and plays a pivotal role in the income security of farmers. The crop is adaptable to diverse environmental conditions and could be cultivated throughout the year and suited for homesteads and as inter-crop. The crop is vulnerable to a number of pests and diseases which limit its production and productivity. Among the various diseases, the viral diseases caused by *Banana bunchy top virus* (BBTV), *Cucumber mosaic virus* (CMV), *Banana streak virus* (BSV) and *Banana bract mosaic virus* (BBrMV) cause major significant yield loss in banana. In addition, banana is also

attacked by nematodes, particularly *Radopholus similis, Pratylenchus coffeae, Meloidogyne incognita* and *Helicotylenchus multicinctus*.

Banana bract mosaic disease was first observed in Philippines in the year 1988 (Magnaye and Espino, 1990). The disease was first reported from Kerala during year the 1996, named as 'Kokkan' in the local language and considered as a disease of unknown etiology (Samraj *et al.*, 1996). Later, Rodini *et al.* (1997) had authentically reported that the Kokkan disease in Nendran banana is caused by *Banana bract mosaic virus* (BBrMV). Now, disease had spread widely in banana growing regions of India *viz.*, Kerala, Tamil Nadu, Karnataka and Andhra Pradesh during the past decade (Singh *et al.*, 2000; Cherian *et al.*, 2002 and Kiranmai *et al.*, 2005). The yield loss caused by BBrMV disease in Kerala was recorded as 70 and 52 per cent in cvs. Robusta and Nendran respectively (Cherian *et al.*, 2002).

BBrMV which belongs to the genus *Potyvirus*, family *Potyviridae*, is the causal agent of banana bract mosaic disease (BBrMD) (Thomas *et al.*, 1997). The particles are flexuous filamentous of size 660-760 x 12 nm with single stranded positive sense RNA genome. The complete genome sequence of BBrMV isolate from Philippines (BBrMV-PHI) was 9711 nucleotides long excluding the 30 terminal poly (A) tail (Ha *et al.*, 2008).

The virus is transmitted by aphids, *Rhopalosiphum maidis* and *Aphis gossypii* in a non-persistent manner (Magnaye and Espino, 1990). Apart from these, other aphid species like *Pentalonia nigronervosa* and *Aphis craccivora* also transmit the virus in non-persistent manner (Selvarajan *et al.*, 2006).

In case of viral disease, early detection and diagnosis of infection is very important since symptomless hosts might carry the viral inoculum. Since banana is a vegetatively propagated plant, there is more chance of carrying latent infection from the mother plants. Disease diagnosis and early detection of the pathogen residing in the planting materials is very important and this could be done through various molecular and serodiagnostic techniques which had been

developed and validated by various virus indexing laboratories. Still more sensitive assays need to be developed. Good quality antiserum is a pre-requisite for serodiagnostic techniques like ELISA. But, the availability of high titre antiserum is the difficulty faced by various virus indexing laboratories.

The present study "Molecular cloning and characterization of coat protein gene of *Banana bract mosaic virus*" would help to develop recombinant clones which have immense application in the field of disease diagnostics and management. These clones could be used for the production of antiserum through recombinant DNA technology and for coat protein mediated resistance in genetic engineering. These could also be used as disease diagnostic probes for more sensitive molecular techniques like Nucleic acid spot hybridization. The objective of the study was to develop molecular clones of coat protein gene (CP) of BBrMV and to characterize it.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Banana is widely cultivated across the globe and prone to many biotic stress caused by different agents. Among the biotic stress, diseases especially viral diseases are the major threat for successful cultivation of banana (Magnaye and Valmayor, 1995). Banana bract mosaic disease (BBrMD) is one of the most important viral diseases of banana which leads to a yield reduction ranging from 52 to 75 per cent (Cherian *et al.*, 2002). The disease is caused by *Banana bract mosaic virus* (BBrMV) which belongs to genus *Potyvirus* and family *Potyviridae*. The virus is transmitted non-persistently by banana aphid (*Pentalonia nigronervosa*) as well as corn aphid (*Rhopalosiphum maidis*), cotton aphid (*Aphis gossypii*) (Espino *et al.*, 1990, Bateson and Dale, 1995).

2.1. OCCURRENCE

The first report of the disease was from Philippines during 1979 (Magnaye and Espino, 1990) and later reported from many countries where banana was grown extensively. The global distribution of the disease is summarized in Table 1.

BBrMD has been reported from many countries in the world including India causing serious yield losses in banana. In India, the BBrMV was first reported during 1966 on plantain cv. Nendran (AAB) as kokkan disease with unknown etiology in the state of Kerala by Samraj *et al.* (1966). Later the causal organism of kokkan disease was confirmed as BBrMV (Rodini *et al.*, 1997). Now, the disease has been reported in major banana growing states of the country *viz.*, Kerala, Tamil Nadu, Karnataka and Andhra Pradesh and identified as a disease of national importance in the country (Rodoni *et al.*, 1997; Selvarajan and Balasubramanian, 2008).

Table 1. Geographical distribution of banana bract mosaic disease

Year of Report	Country	Reference
1979	Philippines	Magnaye and Espino, 1990
1996	Uganda	Tushmereirwe et al., 1996
1996	Ghana	Tushmereirwe et al., 1996
1996	Zanzibar	Tushmereirwe et al., 1996
1996	South Africa	Tushmereirwe et al., 1996
1997	India	Samraj et al., 1966; Rodini et al., 1997
1997	Srilanka	Thomas et al., 1997
1999	Thailand	Rodini et al., 1999
1999	Vietnam	Rodini et al., 1999
1999	Western Samoa	Rodini et al., 1999
2000	Hawaii	Wang et al., 2010
2013	Ecuador	Quito-Avila et al., 2013

The percentage of BBrMD incidence was 4.07, 3.69, 1.39 and 2.22 on different banana varieties *viz.*, Robusta, Mysore Poovan, Ney Poovan and Nendran respectively and Mysore Poovan showed symptoms of both *Banana streak virus* (BSV) and BBrMV (NRCB, 2006).

The disease was noticed on banana plants grown in and around Bangalore and Coimbatore (Singh *et al.*, 1996). The incidence of BBrMD on Nendran was 67.13 per cent in Trichy (Thangavelu and Singh, 1996). Thomas and Magnaye (1996) observed that the disease was widely seen on the varieties of Cardaba (ABB/BBB), Saba (BBB), Abulon (BB) and Embul (AAB, Mysore) and yield loss of up to 40 per cent were recorded in cv. Cardaba and cv. Lakatan (AA) in Philippines. Tushmereirwe *et al.* (1996) reported BBrMV in banana growing regions from Costa Rica and several African countries including Uganda, Ghana, Zanzibar and South Africa.

Incidence of BBrMD at the early growth stage was 44 per cent in malliampathi Nendran orchard. It increased to 58.10 per cent and 60 per cent respectively after bunch emergence and at the time of bunch maturation. More than 50 per cent of Ney Poovan plants in the farms of Agricultural College, Vellayani, Kerala were reported to have severe infection of BBrMV. In the State Govt. Nursery, Peringamala, Kerala, out of 49 cultivars maintained, 14 had severe BBrMV infection. The major cultivars were Mysore poovan, Red banana, Monthan, Rasthali, Jawa, Krishavazhai, Karim kadali, Sakkai, Marti, Mysore Ethan, Padathi, Pisang Lilin and Adukkean. In Ney Poovan the disease incidence was 50 per cent, in Kaliethan it was 6.9 per cent and in Nendran it was 3.4 per cent (NRCB, 1997).

Rodoni *et al.* (1999) detected BBrMV from five different South East Asian countries like Philippines, India, Western Samoa, Vietnam and Thailand. Sharman *et al.* (2000) reported that BBrMV infected Abaca plants in Philippines. Singh and Verma (2001) conducted a survey in Maharashtra and disease incidence was found to be low in the tissue culture banana plantations.

Ariyaraantne and Liyanage (2002) conducted a survey to study the spread of virus disease in various districts of Srilanka and reported that 82 per cent of banana cultivation were infected with BBrMV. The disease caused serious damage to Cavendish types of banana and the yield reduction was up to 70 per cent in Kerala (Cherian *et al.*, 2002).

Selvarajan and Jeyabaskaran (2006) reported the average yield loss due to BBrMV in cv. Nendran (AAB) was 30 per cent. Quito-Avila *et al.* (2013) reported about the occurrence of the disease on Cavendish banana in commercial fields of the province of Guayas, Ecuador for the first time which was confirmed through ELISA and Immunocapture reverse transcription (IC-RT)-PCR.

Incidence of 12 to 30.5% in case of *Banana bunchy top virus* and 2.5% to 40% for *Banana bract mosaic virus* was recorded in Poovan (AAB) grown in Thirukattupalli and Thanjavur district of Tamil Nadu (NRCB, 2015).

2.2. SYMPTOMATOLOGY OF BBrMV

Magnaye and Espino (1990) reported the symptoms of disease as intervening greenish to brownish spindle shaped streaks or long continuous and discontinuous stripes irregularly distributed along the leaf petioles. As the disease progressed, similar discolorations were visible on the bract and fruit bunch. In severe cases of infection, the infected leaves exhibited distinct discontinuous streaks along the primary veins that appeared to be irregularly thickened or raised. Spindle shaped streaks to striped discoloration of varying lengths after removal of dead leaf sheaths were also visible. The name banana bract mosaic was coined because of the conspicuous discoloration and necrotic streaks that developed on the bracts of male bud.

The symptoms were more pronounced on young plants and also on the suckers of affected banana. As the disease advanced, the streaks turned brownish to black and necrotic. Outer leaf sheath showed abnormal detachment from the pseudostem. The affected plants produced small bunches with curved pale green to ash green small fingers. Some of the infected plants showed fan shaped orientation of leaves (Bateson and Dale, 1995).

The name of the virus was derived from the noticeable discoloration and necrotic streaks that developed on the bracts of male bud. Initial symptoms were in the form of greenish to brownish spindle shaped streaks irregularly scattered along the leaf petioles (Magnaye and Valmayor, 1995).

Balakrishnan et al. (1996) reported the detailed affiliation of the disease giving the plants the appearance of 'traveller's palm' with changes in aestivation.

Singh et al. (1996) recorded prominent symptoms of spindle shaped streaks and stripes on the pseudostem and mosaic symptoms on the bracts. In severe cases of infection, the leaves show distinct discontinuous streaks along the primary veins, which were irregularly thickened or raised. Scattered white to yellowish streaks arised from the midrib to the margin of the leaves.

In Tamil Nadu and Maharashtra, BBrMV infected banana plants showed symptoms of typical mosaic and lacked the characteristic symptoms on the bract due to mixed infection of CMV and BBrMV (Rodoni *et al.*, 1997).

Mild spindle shaped mosaic pattern on the upper side and spindle shaped mosaic thick waxy coating on the lower side of the leaves were observed during winter months and during rest of the year, the symptoms were absent. In the same survey, severe necrotic streaks on the pseudostem, petiole and midrib have been observed for the first time in cv. Ney poovan, Robusta and Nendran. On banana var. Pacha bontha, severe necrosis was noted on peduncle, fingers, rachis, bracts and leaf lamina. Mild necrotic streaks became severe at later stages of finger development and cracking of fingers due to necrosis was also noticed (NRCB, 1997).

The red banana variety infected with BBrMV had normal apparent pseudostem with the leaves intact and only the male bud and the fingers showed characteristic mosaic symptoms at lower elevation of Pulney hills (NRCB, 2000).

Singh (2003) observed that the bract of the infected plants exhibited spindle shaped discontinuous dark red streaks. Prominent dark red to purple mosaic streaks were also observed on the pseudostem after the removal of dead leaf sheath. Emerging suckers from infected plant were deeply pigmented. Foliar symptoms appeared as chlorotic streaks parallel to veins. Infected plants produced small bunches on small brittle peduncles along with usual bract mosaic symptoms. The fingers were small and undersized and the size of the bunch was decided by severity of infection (Singh, 2003).

2.3 TRANSMISSION OF BBrMV

The spread and transmission from one host to another is required for virus to survive. However, the presence of cell walls and the immobility of plants are barriers to plant virus transmission. Some viruses are transmitted via seeds or pollen. However, most plant viruses are spread by vectors which include fungi,

nematodes, arthropods and arachnids (Hull, 2002). Mechanical and insect transmission are the most commonly used methods for virus transmission in laboratory or glasshouse studies (Dijkstra and Dejager, 1998).

BBrMV is transmitted in a non-persistent manner by several aphid including *Rhopalosiphum maidis, Aphis gossypii* (Magnaye and Espino, 1990) and *Pentalonia nigronervosa Cocq* (Bateson and Dale, 1995; Thomas and Magnaye, 1996). This virus spreads through vegetative planting materials such as suckers and tissue cultured plantlets but not soil-borne (Thomas and Magnaye, 1996).

Plant viruses are divided into three groups based on the way they are transmitted; non-persistent, semi-persistent and persistent. Non-persistent viruses have short retention time (usually a few minutes to hours) in the vector, in which virus particles are attached to the stylet of the insect and are transmitted to the next plant it probes or feeds on. Persistent viruses remain viable in the insect salivary gland to either replicate (propagative) or not (circulative). Semi-persistent viruses have an intermediate retention time in their vector. Since BBrMV is non-persistent virus with optimum acquisition feeding period and inoculation feeding period for *P. nigronervosa* was 5 min. and 30 min., respectively and efficiency of transmission was 70 per cent (NRCB, 2003). The coat protein (CP) is normally important for virus transmission by insects; however alteration in the CP gene due to mutation could rarely change the transmissibility of the virus (Perry *et al.,* 1998). Almost all viruses including potyviruses like BBrMV encode helper components (HC) to facilitate the association between virions and their vectors (Raccah and Fereres, 2009).

The aphid, *Pentalonia nigronervosa* alone was able to transmit the BBrMV (40 per cent) whereas *Aphis gossypii* and *A. craccivora* failed to transmit the virus. The pre-acquisition fasting period of one hour was effective in the transmission of BBrMV. Short acquisition feeding period of one minute was sufficient for the aphid to become viruliferous. The optimum acquisition-feeding period was found to be 30 min. which gave the maximum per cent of infection

(40%). If the acquisition feeding period was increased beyond 30 minutes the efficiency of vector to transmit the virus was reduced considerably (Dhanya, 2004).

Dhanya (2004) also reported that BBrMV could not be transmitted by sap inoculation through leaf rubbing or sap injection and also not transmitted into local lesion hosts like *Nicotiana tabacum* and *N. benthamina.*

2.4 MOLECULAR DETECTION OF BBrMV

Early detection of disease and information on crop health could make it easy to control disease through proper management practices such as pesticide application to control the vector, disease-specific chemical application; and improve productivity. Detection and identification of viruses, both in plants and vectors, play a major role in virus disease management. The commonly used molecular techniques for disease detection are ELISA and PCR (PCR, RT-PCR and real-time PCR). Other molecular techniques include Immuno Flourescence (IF), Fluorescence *In situ* Hydridization (FISH), DNA microarrays and Flow cytometry (Sindhuja *et al.*, 2010).

2.4.1 Serodiagnostic techniques

2.4.1.1 Direct Antigen Coating Enzyme Linked Immuno Sorbent Assay (ELISA)

ELISA is a sensitive serological technique used for the detection of plant viruses, which is based on antiserum raised in animals against virus-specific proteins. The principle of ELISA is binding of antigens, with a specific enzyme-linked antibody. From a subsequent enzyme substrate reaction, colour or fluorescence obtained, could be elucidated both qualitatively and quantitatively. This technique was introduced in plant virology as an antibody sandwich assay where virus specific antibodies were linked to the enzyme alkaline phosphatase (Voller *et al.*, 1976; Clark and Adams, 1977). It was rapid, extremely sensitive method and could be used to detect viruses in intractable tissues such as the flesh of potato tubers, fruits and the bulbs of bulbous ornamentals (Torrance and

Johnnes, 1981; Cooper and Edwards, 1986). Polyclonal and Monoclonal antibodies are available at both the laboratory and commercial levels against most of the economically important potyviruses and had been extensively used in the detection of potyviruses (Balamuralikrishnan *et al.*, 2002; Desbiez *et al.*, 2002).

Serological methods have been used extensively and successfully to identify different viruses and to determine the relationships between them. However, these methods are not very successful in case of potyviruses (Shukla *et al.*, 1994). This might be due to the dominant epitopes of virions which are the variable N-terminal parts of the virion protein, with a repetitive sequence in some species (Ward *et al.*, 1995), possibly caused by polymerase slippage (Hancock *et al.*, 1995). Although serological tests are often not useful for determining the relationships of potyvirus species, group-specific monoclonal antibodies have been produced that react with the virions of more than 90 percent of tested potyviruses, but not with other potyviruses (Jordan and Hammond, 1991) except *Ryegrass mosaic rymovirus* (Salm *et al.*, 1994). General potyvirus-antisera could be used detect BBrMV infection (Krishna Reddy *et al.*, 2000)

Magnaye and Espino (1990) rapidly screened different banana cultivars for BBrMV using polyclonal antibody sandwich-ELISA technique. It was shown that Abulon and Cardaba were highly infected with *Banana bract mosaic virus* whereas 'K Thong De52' was the least infected among the banana varieties tested.

Rodoni *et al.* (1999) developed F (ab')$_2$ indirect double antibody sandwich ELISA using BBrMV specific polyclonal antiserum. This was used to screen infected banana plants from India and Philippines. They reported that BBrMV specific F (ab')$_2$ DAS ELISA was able to detect BBrMV in the infected sap with dilution up to 1/500. This demonstrated the applicability of ELISA for batch testing and for detecting low concentrations of BBrMV. These results indicated the potential of such diagnostic tests.

National Research Centre for Banana (2000) compared Direct Antigen Coating (DAC)- ELISA and Dot Immuno Binding Assay (DIBA) for the detection BBrMV, from the youngest fully opened leaf midrib. Serodiagnosis done using

DIBA with BBrMV-specific Antibody showed positive reaction in eight samples out of 18 symptomatic samples.

2.4.1.2 Dot Immuno Binding Assay (DIBA)

ELISA is routine method for assaying plant viruses since it is very sensitive. But, the method rather laborious, time consuming and needs sophisticated equipment. However, the technique of immuno blotting (Towbin *et al.*, 1979) was modified to Dot immuno binding assay (Hawkes *et al.*, 1982) in order to simplify the assay. The technique was similar to ELISA except that the plant extracts were spotted onto a membrane rather than using a micro titre plate as the solid support matrix (Hibi and Saito, 1985). In this case, soluble substrate is a chromogenic precipitate which was used for color development and to detect the virus infection by DIBA. Hydrolysis of chromogenic substrate leads to the development of visible colored precipitate at the reaction site on the membrane. Chemiluminescent substrates, which emit light upon hydrolysis and the light signal detected with X-ray film as with radio labelled probes could also be used (Leong *et al.*, 1986). DIBA was reported to be a simple, relatively inexpensive and easy for sample preparation or extraction, and provided information on the distribution of viruses in plant tissues (Lin *et al.*, 1990; Hu *et al.*, 1997).

The demerit of DIBA reported was the possible interference of sap components with the visible diagnostic reactions. Sometimes the color of the sap would prevent weak positive reactions from being observed and the results could not be readily quantified. Nevertheless, their sensitivity, the relatively less assay time needed to index large numbers of samples, the requirement for basic laboratory facilities for the assay, the ability to store blotted membranes for extended periods and less costs made DIBA as useful diagnostic technique for detection of plant viruses. Another advantage is that the samples could be blotted onto the membranes in the field itself and such membranes could be carried or shipped by mail for further processing in a well equipped central laboratory either within the country or abroad (Jain *et al*, 2005).

DIBA was used for the detection of the BBrMV in the infected banana plants and it was reported that among different parts of the plants, youngest fully opened leaf-midrib was most suitable for indexing of BBrMV which showed higher intense reaction than the older leaf's mid ribs (NRCB, 2000).

Dhanya *et al.* (2007) compared the different serological techniques *viz.*, Microprecipitin test, Chloroplast agglutination, Agar gel double diffusion test, Direct Antigen Coating-Enzyme Linked Immunosorbent Assay (DAC-ELISA) and Dot Immuno Binding Assay (DIBA) to evaluate their performance for the detection of virus infection. It was conducted using extracts of infected tissues and antiserum developed against the virus. ELISA and DIBA were found to be more sensitive compared to the other methods.

2.4.2 Nucleic acid based detection technique

The most common nucleic acid-based technique is the Polymerase Chain Reaction (PCR) which was first described in the 1980s by Mullis *et al.* (1986). PCR has become a powerful technique with great impact on molecular biology. It was first used for the amplification of P-globulin genomic sequences for the diagnosis of sickel cell anemia (Saiki *et al.*, 1985) and also employed for the detection and differentiation of plant pathogens (Lopez *et al.*, 2003). Later, it had become a popular technique for the diagnosis of plant viral diseases. The speed, specificity, sensitivity and versatility of PCR made it very suitable among various diagnostic systems. PCR has the potential to amplify even very low concentration of target nucleic acid and form a multiple mixture of different sequences (Martin *et al.*, 2000; Hull, 2002).

2.4.2.1 Reverse Transcription PCR (RT- PCR)

PCR is an *in vitro* method for amplification of target nucleic acid sequences. DNA plant viruses (caulimo, gemini and badnaviruses) could be detected directly by PCR; however, for detection of RNA plant viruses, the RNA needs to be converted to complementary DNA (cDNA) by reverse-transcription prior to PCR.

The cDNA provided suitable DNA target for subsequent amplification and this modified technique for detection of RNA plant virus was called RT-PCR (Reverse Transcription-Polymerase Chain Reaction) (Henson and French, 1993).

Rodini *et al.* (1999) used RT- PCR technique to detect BBrMD in nine different isolates from five countries of South East Asia and reported that RT-PCR assay was most sensitive compared to other detecting methods *viz.*, ELISA and IC-PCR.

Sharman *et al.* (2000) developed multiplex, immunocapture PCR for the simultaneous detection of three viruses from crude sap extracts of banana. The RT-PCR step was required for BBrMV and CMV, because of the presence of ssRNA genomes. The mixed infection of banana viruses including *Banana bunchy top virus* (ssDNA genome) could be detected simultaneously from a single sample.

Hsu *et al.* (2005) designed three degenerate primers, located at the NIb and coat protein gene regions for potyvirus detection. By using these three primer sets, 1.0-1.2 kb cDNA fragments of the 3^1-terminal region of six potyviruses was amplified successfully from infected plant tissues. To identify further, these potyviruse sequences located between the 3^1 end of the NIb gene and the 5^1 end of the coat protein gene was chosen to design a species-specific probes. The results suggested that species-specific cDNA probes were able to identify different species of potyviruse correctly both in single as well as mixed infections.

Sankaralinga *et al.* (2006) designed a set of primers to amplify the coat protein gene of BBrMV with smaller size of 699-bp to detect the virus from infected plant tissues.

Ha *et al.* (2008) designed two pairs of degenerate primers from sequences within the potyviral CI and HC-Pro-coding regions, and these were highly specific to members of the genus *Potyvirus*. Moreover these primers could easily amplify the potyviruses like *Chilli veinal mottle virus* (ChiVMV), *Pepper veinal mottle*

virus (PVMV), *Banana bract mosaic virus* (BBrMV), *Peace lily mosaic virus* (PeLMV), *Telosma mosaic virus* (TelMV) and *Wild tomato mosaic virus* (WTMV).

Caruana *et al.* (2008) developed Immunocapture (IC) one-step RT-PCR assay to detect BBrMV in single and also in bulk samples of banana plants. Primer sets of Bract N1/NR and N2/NR which were specific to BBrMV were designed and used in RT-PCR and IC-RT-PCR assays, where the RT and PCR reaction were conducted simultaneously in the same tube.

2.4.2.1.1 Primer designing

Primers are single stranded oligonucleotide sequences work in pairs, forward primer and reverse primer except in case of Rapid amplification of polymorphic DNA (RAPD). The primer pairs are chosen to extend towards each other to cover the given target region (Kampke *et al.*, 2001). Designing of appropriate primer is necessary in PCR, DNA sequencing and hybridization. While designing a primer, two main goals are to be balanced; the specificity of primers to avoid mispriming, and the efficiency of primers to be able to amplify a product exponentially (Dieffenbach *et al.*, 1993). Usually primer of 20-24 bases and GC content between 45-60 per cent with T_m of 52-58°C works best in most applications. The annealing temperature is generally calculated as 5°C lower than the estimated melting temperature (T_m). Within a primer pair, the GC content and T_m value should be well matched (Elsalam, 2003).

High G/C content in the 3'end of primer is not desirable as it would lead to mispriming. However, "G" or "C" is desirable at the 3' end of primers since this would reduce breathing and thereby increase yield (Sheffield *et al.*, 1989; Elsalam, 2003). When designing primers, it is important to have a minimum molecular homology between primer set to inhibit the formation of either hairpins or primer dimers (Elsalam, 2003).

2.5.1 Biological characterization

BBrMV is a flexuous, filamentous virus particle that belonged to the genus *Potyvirus* and family *Potyviridae* (Magnaye and Valmayor, 1995). BBrMV was described as flexuous virion measuring 760x12 nm which is transmitted non-persistently through several aphid species *viz. Pentalonia nigronervosa, Rhopalosiphum maidis* and *Aphis gossypii* (Bateson and Dale, 1995).

Thomas *et al.* (1997) purified virions of a Philippine isolate of BBrMV from field-infected banana cv. Cardaba. Particles were nearly 725-nm long, banded at a density equivalent to 1.29 to 1.31 g/ml in cesium chloride equilibrium gradients, and had absorbance value of 1.17 *at* $A_{260/280}$. Yield of about 4 mg/kg from fresh or frozen leaf midrib or lamina tissue were obtained. Three major protein species with sizes of 31, 37, and 39 kDa were resolved from dissociated virions, and all reacted specifically with polyclonal antibodies.

The family *Potyviridae* is the largest family of positive-sense, single-stranded RNA (ssRNA) plant virus. The uniqueness of the family induced the formation of 3D Crystalline cytoplasmic inclusions (CI) within the infected cells (Shukla *et al.*, 1998). The members of the family are divided based on their transmission vectors and genomic characteristics, into six genera namely, *Potyvirus, Ipomovirus, Macluravirus, Tritimovirus, Bymovirus and Rymovirus,* and the largest being the genus *Potyvirus which* contained 158 viral species including BBrMV (Berger *et al.*, 2005). Distinguishable feature of these viruses was its transmission by aphids in a non-persistent manner.

Based on the sequence of C-terminal, half of the coat protein coding region and the 3'-untranslated region (UTR), BBrMV was shown to be a distinct *Potyvirus* with contained 38-k Da coat protein that reacted with polyclonal *Potyvirus* antiserum (Bateson and Dale, 1995; Thomas *et al.*, 1997).

2.5.2 Molecular characterization

The genome of BBrMV was characterized by encapsulated a monopartite ssRNA genome of length 9711 bp yielding for a poly protein of 3,125 amino acids. The virus contains a typical open reading frame (ORF) linked to 5'untranslated region (5'UTR) and a 3'UTR region terminating in a poly adenylated (polyA) tail. The ORF encoded a single large polyprotein that was co-translationally processed into ten functional matured proteins; namely, the first protein (P1), helper component protease (HC-Pro), third protein (P3), 6K1, cylindrical inclusion protein (CI), 6K2, small nuclear inclusion protein (NIa; including the VPg and protease (NIa-Pro) domains), large nuclear inclusion protein (NIb; replicase) and coat protein (CP) that had conserved motifs among homologous protein of other potyviruses (Balasubramanian and Selvarajan, 2012).

The coat protein (CP) is a well characterized protein of *Potyvirus* having multiple functions; involved in cell-to-cell movement, regulate amplification of viral RNA, encapsidation of the RNA, vector transmission and also host specificity (Urcuqui, 2001). CP roughly divided into three domains; the N domain which is highly variable contains the major virus specific epitopes, the C domains and the core which are conserved. The variation in the core region was similar to that of the whole genome and therefore, is a reliable index for genetic relatedness (Shukla *et al.*, 1998).

Information on variability among populations of plant RNA viruses is necessary to study the evolution and their interactions with hosts (Arena *et al.*, 2001). Sequence comparison of CP gene provides evolutionary relationship between strains of potyvirus and to determine and differentiate distinct potyviruses (Shukla *et al.*, 1989).

The coat protein N- terminal region that is exposed on the virion surface contained a highly conserved DAG motif situated close to the N- terminus. Site directed mutagenesis analyses revealed that the motif was required for aphid transmission (Atreya *et al.*, 1995). Mutation analysis by Dolja *et al.* (1994)

showed that the N- and C- terminal regions of *Tobacco etch virus* (TEV) CP were essential for systemic viral movement, where as the core region was required for cell to cell movement. Later on, it was found that upholding of the CP N-terminal neutralized net charge, but not primary sequence, was prerequisite for systemic infection of *Zucchini yellow mosaic virus* (ZYMV) (Kimalov *et al.*, 2004). Even though initial studies suggested that CP N and C- terminal regions were not necessary for particle assembly (Shukla *et al.*, 1994; Voloudakis *et al.*, 2004), later studies showed both regions were necessary for assembly of virions (Anindya and Savithri, 2003; Kang *et al.*, 2006). The interaction between the CP and the Nib through the GDD motif of Nib suggested that the CP could be involved in regulation of RNA synthesis (Hong *et al.*, 1995).

2.5.2.1 *Cloning and Sequence analysis*

RNA cloning is usually done by reverse transcription into cDNA followed by insertion into plasmid or lambda vectors and transformation of strains of *E. coli* (Maniatis *et al.*, 1982). Molecular cloning of many viruses infecting plant has been carried out. The main objective of cloning plant viruses has been the improvement of virus detection and diagnosis (Jelkmann *et al.*, 1989).

Many potyvirus RNAs had been partially or completely cloned (Nagel and Hiebert, 1985). The complete genome sequences of isolates BBrMV- PHI from Philippines (Ha *et al.*, 2008) and BBrMV- TRY from India (Balasubramanian and Selvarajan, 2012) were determined.

Bateson and Dale (1995) amplified the coat protein gene of BBrMV of Philippines isolates using a potyvirus specific degenerate primer which was cloned, sequenced and analyzed. The Open reading frame (ORF) of amino acid (aa) sequence was most similar to the C-terminal half of the *Maize dwarf mosaic potyvirus* coat protein and the BBrMV 3′ untranslated region was similar to the *Ornithogalum mosaic potyvirus*.

Rodini *et al.,* (1999) amplified BBrMV with 604 bp product including the CP-coding region and 3' untranslated region (UTR) region of genome by using specific primers. The amplified product was cloned and sequenced. The sequence analysis revealed that no phylogenetic relationship existed among isolates.

Sankaralingam *et al.* (2006) amplified the coat protein (CP) gene of Indian BBrMV isolate which consisted of 1062 nucleotides (nt) including 900 nt of the CP coding region and 162 nt from the 3' UTR. When the sequence of the coat protein gene was compared with other Indian isolates variability of 1.0-4.6 per cent at nucleic acid level and 0.7-2.0 per cent at the amino acid level was observed. Variability between Indian isolates and the four South East Asian isolates, ranged from 4.5-5.3 per cent at nucleotides and 1.4-3.0 per cent at amino acid levels, respectively.

Wang *et al.* (2010) firstly reported the incidence of BBrMV in flowering ginger, *Alpinia purpurata* (Vieill.) K. Schum., which was a popular cut flower plant in Hawaii with symptoms including severe mosaic and stripes on the leaves, cupping and browning of flowers and caused reduction in size and shelf life. BBrMV was detected using RT-PCR with coat protein gene specific primers to amplify a 279-bp conserved region of the coat protein gene of BBrMV and sequence analysis of cloned product showed 99 per cent identical to corresponding sequences of BBrMV

Siljo *et al.* (2012) reported a new viral disease expressing symptoms of the chlorotic streaks on veins of cardamom leaves in plantations of Kerala, Karnataka and Tamil Nadu. When RNA extracted from these infected plants subjected to RT-PCR using primers specific to conserved region in Potyvirus produced an amplicon size ~700 bp and showed that BBrMV was the closest virus using BLAST analysis of this amplicon sequence. The analysis of the coat protein gene sequence showed about 94 per cent identity with BBrMV isolates and about 60 per cent identity with other distinct potyvirus species which indicated that the causal virus was a strain of BBrMV.

Balasubramanian and Selvarajan (2012) amplified, cloned and sequenced the complete genome of an Indian isolate (TRY) of BBrMV from the French plantain cv. Nendran (AAB). The complete genome of BBrMV consisted of 9711 nucleotides excluding the poly(A) tail and these genome organization had similarity to that of Philippine (PHI) isolate which was characterized earlier. The phylogenetic analysis suggested that the BBrMV-TRY isolate was closely related to the BBrMV-PHI isolate with 94 per cent similarity.

Balasubramanian *et al.* (2014) amplified, cloned and sequenced the helper component proteinase (HC-Pro) gene of 22 BBrMV isolates from India and reported that sequence identity with other previously reported BBrMV isolates was 92-100 per cent both at the nucleotide and amino acid level. Phylogenetic analysis based on nucleotide sequences of non-recombinant isolates showed that the isolate TN15, TN9 and TN24 formed one cluster and all the remaining isolates in another cluster. Different functional motifs in the central region of HC-Pro gene of BBrMV isolates were found to be conserved. Four potential recombinants with 15 breakpoints were observed at the N and a few from C terminal regions compared with other isolates

Balasubramanian and Selvarajan (2014) investigated the global genetic diversity and molecular evolution of BBrMV coat protein gene. Multiple alignments of coat protein gene of 49 BBrMV isolates showed that the nucleotide and amino acid identity of about 79-100 and 80-100 per cent respectively. Phylogenetic analysis also revealed that except the two Indian isolates, TN14 and TN16, all isolates clustered together in one group. Eleven recombination events were also detected in 49 BBrMV isolate using Recombination detection program. Maximum-likelihood methods revealed that most of the codons in the coat protein gene were under negative selection except for codons 28, 43, and 92 which were under positive selection.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The present study on "Molecular cloning and characterization of coat protein gene (CP) of *Banana bract mosaic virus*" was carried out at Centre for Plant Biotechnology and Molecular Biology (CPBMB) and Department of Plant Pathology, College of Horticulture, Vellanikkara during 2015-2016. The details of materials used and the methodologies followed are described in this chapter. The details of the laboratory equipments used in the study are provided in Annexure-I.

3.1 SYMPTOMATOLOGY

The types of symptoms expressed on different parts of the plant *viz.*, leaves, pseudostem, male-bud (bract) and bunches associated with *Banana bract mosaic virus* (BBrMV) infection were monitored and documented under natural field conditions.

3.2 COLLECTION AND MAINTENANCE OF VIRUS CULTURE

BBrMV infected plants showing distinct symptoms were collected and maintained as virus culture in Insect proof net house, Department of Plant Pathology, College of Horticulture, Vellanikkara. Healthy tissue culture plants of banana were also maintained separately and used for various studies.

The infected samples were screened to confirm the presence of virus by protein based detection *viz.*, Direct Antigen Coating ELISA (DAC-ELISA), Dot Immuno Binding Assay (DIBA) and nucleic acid based detection like RT-PCR were performed. The young leaves of healthy and infected plants were collected from net house and stored at -80^0 for further studies.

3.3 DETECTION OF BBrMV BY PROTEIN BASED METHODS

3.3.1 Direct Antigen Coating ELISA (DAC-ELISA)

Protein based detection method *viz.*, DAC-ELISA was validated. Infected leaf sample of banana showing typical symptoms and healthy control were tested

for BBrMV infection by Direct Antigen Coating ELISA (DAC- ELISA) using monoclonal antibody obtained from Agdida, USA Ltd.

3.3.1.1 Determination of titre of antiserum

DAC-ELISA was performed with different dilution of (1:100; 1:200; 1:300; 1:500) antiserum to determine the antiserum titre for monoclonal antibody specific to BBrMV using procedure described by the Dhanya et al., 2007.

Antigen was prepared by grinding the leaf samples in coating buffer (Annexure II) using chilled pestle-mortar and centrifuged the homogenized product at 15000 rpm for 10 min at 4° C. Wells of microtitre plates (with 96 wells, Tarsons Products Pvt. Ltd) were coated with 100 µl of supernatant (prepared antigen) and incubated for 2 h at 37 °C temperature. After incubation the plates were washed thrice with Phosphate buffered saline with tween-20 (PBS-T) buffer (Annexure II) to remove the sap and unbound antigen at three minutes intervals followed by gently tapping the plates to remove the residual liquid. 100 µl of blocking solution (Annexure II) was added to each well and incubated at 37 °C for one hour and then washed in PBS-T washing buffer. Antiserum (100 µl) with different dilutions (1:100, 1:200, 1:300 and 1:500) in antibody buffer was added to respective wells and incubated overnight at 4 °C. The plates were then washed in PBS-T buffer and 100 µl of secondary antibody conjugated with alkaline phosphatase (1:500 dilution) was added to each well and incubated at 37 °C for 2 hours. The unbound secondary antibodies were washed with PBS-T and freshly prepared 100 µl substrate p-Nitrophenyl phosphate (Annexure II) was added to each well and incubated in dark at 37 °C for 30 min for color development. The absorbance value was recorded at 405 nm by ELISA reader (VESAMAX Microtitre plate reader). The optimum dilution was determined based on the absorbance value recorded.

3.3.1.2 Detection of BBrMV by DAC-ELISA

Once the primary antiserum titre was standardized, DAC-ELISA was again performed using the standardized titre of primary antibody (1:200), which was previously determined in the section 3.3.1.1. The absorbance was measured at 405 nm using VERSAMAX ELISA reader. The absorbance values of the test sample and healthy sample along with buffer control were compared and if the absorbance value of test sample was more than twice that of healthy sample then the sample was considered as positive for virus infection.

3.3.2 Detection of BBrMV by DIBA

DIBA was done using procedure described by Banttari and Goodwin (1985) with slight modification. A desired size of Nitrocellulose membrane was cut and marked one cm^2 on it. The membrane was washed with distilled water and air dried. 2 μl of sample containing crude antigen was spotted on the marked square and air dried for 15 min.

After drying, membrane was immersed in blocking solution (Annexure II) with gently shaking for one hour. Then it was washed three times with PBS-T at 3 min. intervals. Primary antibody solution (Monoclonal antibody from Agdida, USA Ltd with 1: 200 dilution) was then added on blot, incubated for 2 h at room temperature and followed by washing with PBS- T buffer thrice at 3 min. interval. Secondary antibody (Agdida, USA Ltd) conjugated with alkaline phosphatase was added on blot, incubated for one hour followed by three times (5 min each) wash with PBS-T buffer. Finally, membrane was rinsed in substrate solution (Annexure II) and incubated under dark condition for 15-20 min. Then the membrane was washed with distilled water, air dried and observed for colour development.

3.4 STANDARDIZATION OF NUCLEIC ACID BASED DETECTION METHOD

For detection of BBrMV in leaf sample, RT-PCR was performed with RNA isolated from leaf samples using species specific primers.

3.4.1 Isolation of RNA

Leaf samples of virus infected plants showing positive result in ELISA and DIBA along with healthy sample were taken for RNA isolation. RNA was isolated using Ambion PureLink ® Plant RNA reagent (Thermo Scientific) and TRIzol based method (Sigma Aldrich). RNA isolated from both the methods were compared, analyzed and stored at -20^0 C. All the materials used for RNA isolation were soaked in 0.05 percent (v/v) DEPC treated water overnight and autoclaved.

3.4.1.1 RNA isolation by TRIzol based method

RNA was isolated from leaf sample by manual method using TRIzol reagent (Sigma Aldrich). 100 mg leaf sample was ground using liquid nitrogen in a sterile DEPC treated pestle and mortar. The powdered tissue was transferred to RNase free micro centrifuge tube and was homogenized with 1000 μl of TRI reagent. It was centrifuged at 13,000 rpm (Kubota-3500) for 15 min at 4°C and the supernatant was transferred to a fresh centrifuge tube and kept for 5 min at 35^0 C. To the supernatant, 200 μl of chloroform was added, shaken vigorously and incubated for 30 min at room temperature. Three separate phases were formed when centrifuged at 13,000 rpm for 10 min at 4°C. Out of the three layers, colourless upper phase which contained of RNA was transferred into a fresh micro centrifuge tube and 500 μl of Isopropanol was added. Then it was incubated for 30 min at 35^0 C and centrifuged at 13,000 rpm for a period of 10 min at 4°C. The supernatant was discarded and the pellet was washed with 75 per cent ethanol by gently vortexing followed by centrifugation at 7500 rpm for 5 min. Finally, air dried the pellet for 15 min and resuspended in nuclease free water.

3.4.1.2 RNA isolation by Ambion PureLink ® Plant RNA reagent method (Thermo Scientific)

100mg leaf sample was taken and ground to fine powder in liquid nitrogen using sterile DEPC treated pestle and mortar. The tissue powder was transferred into RNase-free micro centrifuge tubes, homogenized with 500 μl cold (4°C)

PureLink® Plant RNA Reagent and incubated for 5 minutes at room temperature and incubation was done by laying the tube down horizontally to maximize surface area. This was clarified by centrifuging at 12,000 rpm in for 2 min at room temperature and transferred clarified solution to a clean RNase-free micro centrifuge tube. 100 µl NaCl (5M) and 300 µl chloroform were added to clarified solution and mixed thoroughly by inverting tube. Then it was centrifuged at 12,000 rpm for 10 min at 4°C. After centrifugation three layers were formed. The upper, aqueous phase was transferred to a clean RNase-free tube and equal volume of Isopropanol was added. After 10 minute incubation at room temperature, it was centrifuged at 12,000 rpm for 10 min at 4°C and discarded the supernatant. To the pellet, 1000 µl of 75% ethanol added and centrifuged at 12,000 rpm for one min. Finally, the pellet was air dried and resuspended in 30 µl of nuclease free water.

3.4.1.3 Agarose gel electrophoresis

The method used to determine the quality of RNA is to run an aliquot of the sample on agarose gel. Agarose gel (2%) was prepared in IX TAE buffer (Annexure III) and ethidium bromide was added to a final concentration of 0.5 µg ml^{-1}. An aliquot of the RNA sample (5 µl) mixed with 1 µl loading dye was loaded in each of the wells of the gel. The electrophoresis was carried out at 50V (BIO RAD Power Pac HV, USA) for 30 min. The gel was then visualized and the image was documented using Alpha imager (UVP Geldoc IT- Imaging system,U.K).

3.4.1.4 Spectrophotometer analysis

The quantity and quality of RNA was recorded by determining the absorbance of RNA samples. The optical density (OD) of RNA samples was measured using NanoDrop1000 spectrophotometer (Thermo Scientific, USA). The spectrophotometer was calibrated to blank (zero absorbance) with one µl of nuclease free sterile water. Then, the concentration of the RNA samples (1 µl

each) was recorded. The quality of RNA preparation was determined by analyzing the absorbance value at the $A_{260/280}$.

3.4.2 Synthesis of first strand complementary DNA (c-DNA)

RNA isolated was used to synthesis the c-DNA by using RevertAid H Minus First Strand cDNA synthesis kit (Thermo Scientific) as per manufacturer's instructions. The cDNA synthesis was carried out in PCR (Agilent Technologies, USA). The components of reaction mixture used are given in the Table 2.

Table 2: Components of reaction mixture for cDNA synthesis

SI. No.	Name of the component	Volume (µl)
1	RNA	2
2	Oligo (dT)$_{18}$ primer	1
3	Water (nuclease free)	9
4	10x Reaction buffer with $MgCl_2$	4
5	RiboLock RNase Inhibitor (20U/ µl)	1
6	RevertAid H Minus MuLV RT (200U/ µl)	1
	Total volume	**20**

The template RNA, reverse primer and nuclease free water were added into a sterile polypropylene PCR tube and mixture was incubated at 70°C for 5 min in thermocycler. After incubation, it was immediately chilled on ice for 2 min and then remaining components were added. Finally the mixture was incubated at 42°C for 60 min followed by incubation at 70°C for 10 min in the thermo cycler.

3.4.3 Standardization of annealing temperature

The annealing temperature of two primer sets obtained from SIGMA-ALDRICH were standardized in the present study.

1. Bract 1(B1) and Bract 2(B2) primers (28bp) specific to coat protein of BBrMV were synthesized based on the partial CP sequences and 3' Untranslated region

(UTR region) (Rodini *et al.,* 1999) and were used to identify virus infection with partial coat protein amplification.

B1 (forward primer) sequence: 5'GACATCACCAAATTTGAATGGCACATG G 3'

B2 (reverse primer) sequence: 5'CCATTATCACTCGATCAATACCTCACAG 3'

2. BCPF1 and BCPRl (18bp) primers were designed (As in the section 3.5.1) and synthesized to amplify coat protein gene.

BCPFl sequence: 5' GATGATGACCCAAGCCGC 3'

BCPRl sequence: 5' GCAGAGAG GCATATCAC 3'

In order to standardize annealing temperature of primer pairs used, Gradient PCR was carried out. The cDNA synthesized from the RNA of infected leaf samples were used for PCR amplification. The PCR reaction mixture was optimized as given in Table 3.

After the preparation of the reaction mix, it was vortexed. PCR was carried out in Thermocycler (Agilent Technologies, USA) using programme set at an initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 45s, annealing temperature gradient from 48°C to 60°C for one min. and extension at 72 °C for one min. The final extension was carried out at 72 °C for 10 min. Control reactions with healthy samples were carried out to distinguish the target products from non-target products. The PCR amplified products along with 100 bp plus DNA ladder (Thermo Scientific. USA) were separated on agarose gel (1.5%). The gel was viewed using gel Imager. The best annealing temperature was selected based on the intensity of amplification.

Table 3: Components of reaction mixture used for gradient PCR

Sl No.	Name of component	Volume (µl)
1	10X PCR buffer	2.5
2	25 mM MgCl$_2$	2
3	20 mM dNTPs mix	2
4	Taq polymerase	0.5
5	Primer (0.3µlF+ 0.3µlR)	0.6
6	c-DNA	3
7	Water	14.4
	Total volume	**25**

3.4.4 Uncoupled RT-PCR

Uncoupled RT-PCR is one of the approaches of RT-PCR using separate reactions for cDNA synthesis and PCR. Once the annealing temperatures were standardized for the primer pairs, the cDNA samples were subjected to uncoupled RT-PCR using same reaction mixture as described in section 3.4.3. PCR was carried out in Thermo Cycler (Agilent Technologies, USA) and the same cycling conditions were set as given in section 3.4.3 with an annealing temperature of 58 °C for B1 and B2 primers. The PCR products were analyzed on 1.5% agarose gel. The amplicons showing expected band size were purified by gel elution method.

3.4.5 Extraction of PCR product from gel

The amplicons showing expected band size were purified using GenElute^{TM} Gel Extraction Kit (Sigma Aldrich) by gel elution method as per manufacturer's instruction. The gel slice containing the amplified DNA fragment of size 605 bp was excised using a clean sterile scalpel, it was placed into a pre-weighed 1.5 ml eppendorf tube and weighed again to determine the weight of gel slice. Binding buffer was added to the gel slice in ratio of 1:1 (Buffer volume: Gel weight) and mixture was incubated 50-60^0 C in dry bath for 10-15 min. Once it dissolved completely; the solution was transferred to the purification column and

centrifuged for one minute at 12,000 rpm. The flow through was discarded and the column was placed back into the same collection tube and centrifuged at 12,000 rpm for one min by adding 700 µl of wash buffer. Again flow through was discarded and centrifugation was done again at 12,000 rpm for one min to remove the residual wash buffer. Finally, the elution buffer was added to purification columns which were transferred into clean micro centrifuge tubes and centrifuged at 12000 rpm for one min. The columns were discarded and the purified DNA was stored at -20^0 C.

3.4.6 Reamplification of gel eluted products

Since PCR amplified product (~605 bp size) by *Taq* polymerase generated the complementary 3' overhang end which allowed the hybridization with vector, the eluted product obtained was reamplified using the same reaction mixture used in section 3.3.2.3 (except that instead of cDNA, gel eluted product was used) under similar cycling conditions. Quality of amplified products was checked on agarose gel (1.5%) and same product was used for cloning.

3.4.7 DNA sequencing

The eluted product was cloned into pGEM®-T vector as described in section 3.7. The products cloned were sequenced at the SciGenome, Regional centre Kochi. Nucleotide BLAST of the obtained sequences was performed in order to find out the similar sequences.

3.5 AMPLIFICATION OF COAT PROTEIN (CP) REGION OF VIRUS USING DESIGNED PRIMER

For amplification of coat protein (CP) region, a set of primers were designed using sequence obtained from NCBI genbank.

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3.5.1 Primer designing

The complete CP gene sequences of BBrMV available in NCBI were selected in FASTA format and the details of selected sequences are provided in Table 4. The multiple sequence alignment of nucleotide sequences obtained was done using Clustal omega' (http://www.ebi.ac.uk/Tools/msa/clustalo/; Thompson *et al*, 1994) (Figure 1). Based on the homology, conserved boxes of 18 to 24 bases each for both primers were selected along the sequence and the forward and reverse primers were designed from those conserved boxes based on ideal primer parameters given below.

- Primer with GC content not less than 50 per cent
- Melting temperature ($Tm = 4GC + 2AT$) ranges between 60°C and 70°C.
- The distance between the primers ranges from 500 to 1000 base pairs.
- GC content at 3' end.
- Without any complementarity between forward and reverse primers.
- Avoidance of repeats of single base within the primer sequence.
- The distance between forward and reverse primer was greater than 500bp.
- Length of each primer was 18 to 24 bp long.

Based on these characters, the sequence of the forward primer was taken as such and for the reverse primer, the reverse complementary sequence was taken.

Table 4: Details of sequences used for primer designing

Sl. No.	Acession No	Isolate name	Source	Size (bp)	Reference
1	EU009210	Trichy	Tamil Nadu	914	Selvarajan and Balasubramanian, unpublished data, 2007; unreferenced
2	KF385480	AP7	Andhra Pradesh	900	

Table 4 Continued…

3	KF385483	AS2	Assam	900	Balasubramanian and Selvarajan,2014
4	KF385491	KER 2	Kerala	900	
5	KF385490	KAR 3	Karnataka	900	
6	KF385473	TN9	Tamil Nadu	900	

3.5.1.1 Primer evaluation

In advance to synthesis of the primers, *in-silico* analysis was carried out to determine the specificity and other characteristics like the length of primers ,the self-complementarity, expected PCR product, GC content, melting temperature, feasible annealing temperature and probability for primer dimer formation, hairpin formation were assessed using OligoAnalyzer 3.1 (Integrated DNA technologies) available at http://eu.idtdna.com/site (Figure 2). The primer sequences thus validated by *in silico* methods were sent to Sigma Aldrich (USA) for synthesis.
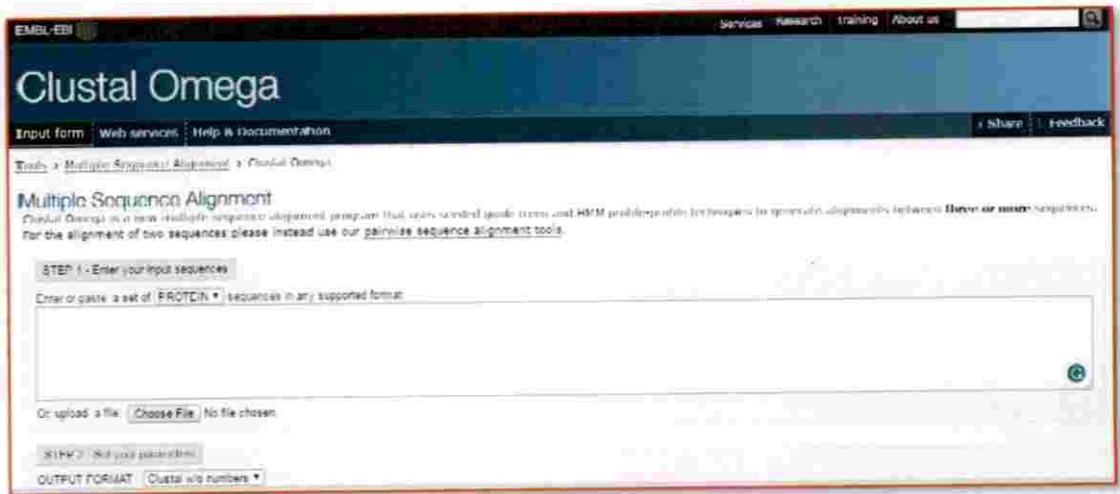
Figure 1: Clustal Omega tool used for sequence alignment to design primer
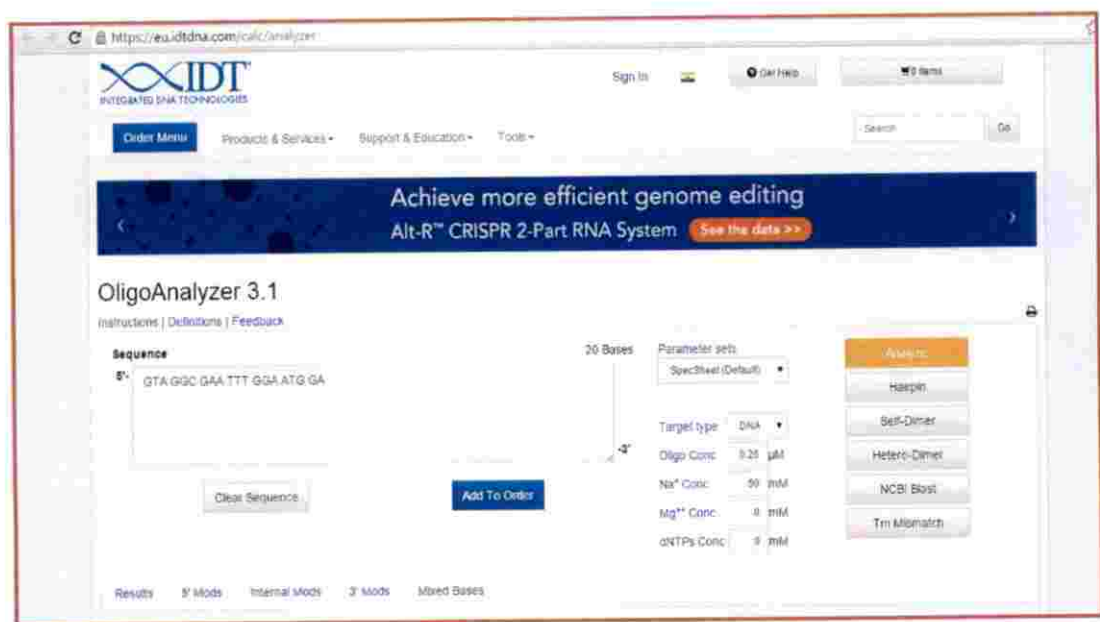
Figure 2: OligoAnalyzer 3.1 used for validation of primers

3.5.2 Uncoupled RT-PCR using designed primer

Once annealing temperature was standardized for the newly designed primer pair, uncoupled RT-PCR was done with the cDNA samples using the reaction mixture as procedure described in section 3.4.3 PCR was carried out in Agilent Technologies Thermo Cycler (USA) and the same cycling conditions were set as given in section 3.4.3 with the standardized annealing temperature of 55.6 °C for BCPF1 and BCPR1 primers. The PCR products were analyzed on 1.5 per cent agarose gel. The amplicons showing expected band size of 850 bp were excised and purified by gel elution method as described in section 3.4.5. The eluted DNA was reamplified as described in section 3.4.6 and used for cloning.

3.5.3 Cloning of coat protein (CP) gene

3.5.3.1 Preparation of Competent Cells

The competent cells (*Escherichia coli* DH5α) were prepared for plasmid transformation by following the protocol described by Mandel and Higa (1970). The steps followed for competent cell preparation are as follows:

Day 1:

- Single colony of 18 h old *Escherichia coli* DH5α strain was inoculated to 100 ml LB medium under sterile condition and incubated overnight at 37°C on a shaker (Lab companion S1-600) set at 160 rpm.

Day 2:

- Three ml of the culture was transferred aseptically to 50 ml sterile LB broth and incubated for 4 h at 37^0 C on a shaker set at 160 rpm.
- The cells were then aseptically transferred to sterile disposable ice-cold 50 ml tube.
- The culture was cooled on ice cubes for 20 min.
- The cell suspension was centrifuged at 3500 rpm for 10 min at 4°C
- The supernatant obtained was carefully discarded and the pellet was gently resuspended in 10 ml sterile ice-cold 0.1M $CaCl_2$.

- The tubes were kept on ice for 20 min and the cell suspension was centrifuged at 5000 rpm for 10 min at 4°C.
- The supernatant was decanted and the pellet was resuspended in 2 ml of ice cold sterilized 0.1M $CaCl_2$. The tubes were kept on ice for 18 h.

Day 3:

- Four ml of chilled glycerol was added to the cell suspension and mixed well using a sterile microtip.
- The competent cells were prepared as aliquots of 100 ul in chilled 1.5 ml microfuge tubes and covered with aluminum foil. These are stored at -80°C for further use.

3.5.3.2 Screening of competent cells

Transformation of competent cells with a plasmid having ampicillin resistance (pUC18) was carried out to check the competence and purity of competent cells. The procedure followed for screening of plasmid is as follows,

- The competent cells stored at -80°C were thawed over ice for 10 min.
- 2 μl of plasmid DNA was added to 100 μl competent cells. Competent cells without adding plasmid as negative control was placed simultaneously.
- The cells were kept on ice for 40 min. Heat shock was given at 42°C for 90 sec in a water bath and then kept on ice for 5 min.
- LB medium (250 μl) was added to the cells and incubated at 37°C for one hour on a shaker set at 120 rpm.
- 100 μl of transformed cells were plated on LBA/ampicillin (Annexure IV) and incubated overnight at 37°C in a shaker (100 rpm). The recombinant clones were grown on plates containing ampicillin.

3.5.3.3 Ligation

Ligation was carried out to link the DNA insert and the plasmid vector. The eluted amplified product was ligated in pGEM-T Easy Vector System (Figure 3) (Promega Corporation, USA) as per manufacturer's protocol mentioned below;
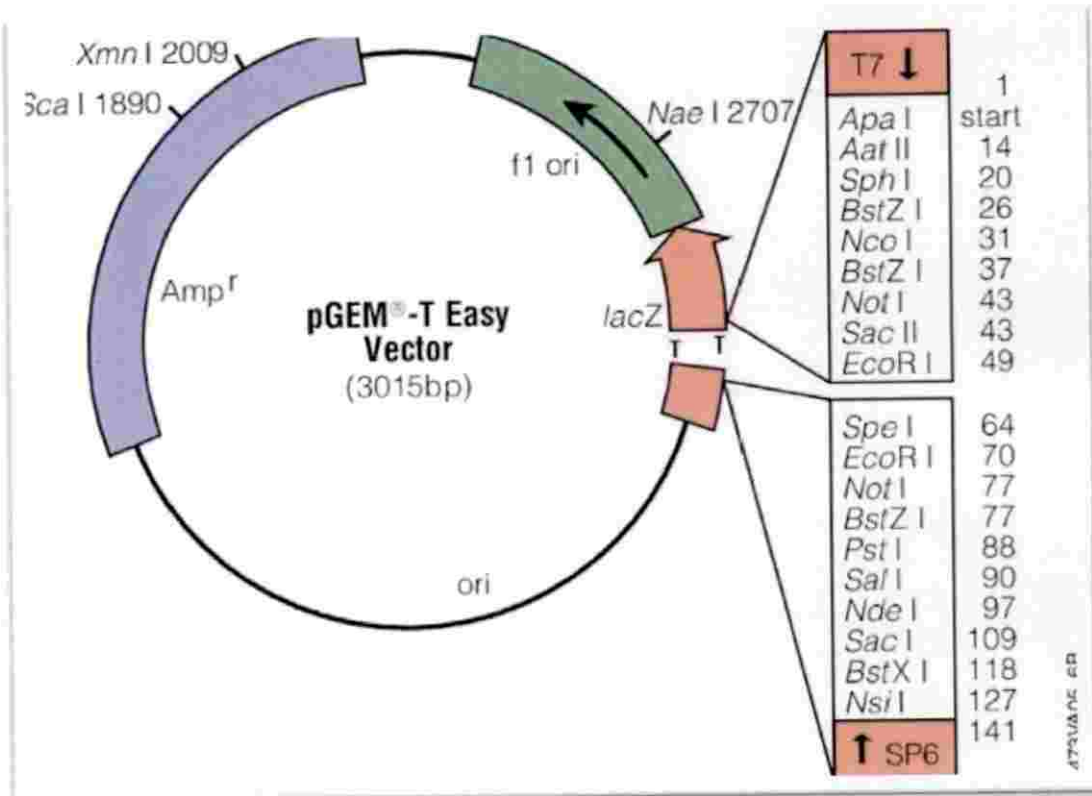
Figure 3: Restriction map showing multiple cloning site of vector pGEM-T vector (Promega) used for cloning.

before use, the vector and other components were briefly centrifuged to collect the contents at bottoms of the tube. The ligation reaction was set by mixing the reaction components mentioned in table 5 by pipetting and incubated the reactions overnight at 4°C.

Table 5: Components of ligation reaction

Sl. No.	Components	Volume (µl)
1	2X rapid ligation buffer	5
2	pGEM-T Easy Vector(50ng)	1
3	DNA product	3
4	T$_4$DNAligase (3units/µl)	1
	Total volume	**10**

3.5.3.4 Transformation

Transformation of competence cells with pGEM-T Vector plasmid was performed. The ligated PCR product was added to 100µl of thawed competent cells and kept on ice for 30 min. Heat shock was given at 42°C for 90 seconds in a dry bath and immediately replaced on ice for 5 min. 250µl of LB broth was added to the cells and incubated at 37°C for one hour in a shaker at 1600 rpm. The aliquots of transformed cells were placed on LB agar/ampicillin/ IPTG /X-gal plates and incubated overnight at 37°C. The transformed colonies were selected based on blue/white selection and further confirmation of presence of insert was done by colony PCR.

3.5.3.5 Colony PCR; Confirmation of presence of insert

Single white coloured colonies were selected and resuspended in the 20 µl of distilled water. Then it was heated at 98° C for 3 min followed by centrifugation at 12000 rpm for 2 min. The supernatant was taken in PCR tube and was used as template DNA for PCR reaction. The recombinant clones were analyzed for the presence and orientation of the DNA insert by colony PCR using plasmid primers T_7 and Sp_6 (Promega, USA).The PCR reaction mixture was formulated as given in Table 6.

Table 6: Components of colony PCR reaction

SI No	Components	Volume (µl)
1	DNA	2.0
2	10 x reaction buffer	2.5
3	$MgCl_2$	1.25
4	dNTP (10mM)	2.0
5	Forward primer T_7 (10pM/ µl)	0.5
6	Reverse primer Sp 6 (10pM/ µl)	0.5
7	*Taq* polymerase (3U/ µl)	0.2
8	Sterile distilled water	16.05
	Total volume	**25**

The PCR was performed with an initial denaturation at 94 °C for two min, followed by 30 cycles of denaturation at 94 °C for 45s, annealing at 55 °C for 1min and extension at 72 °C for one min. The final extension step for 10 min at 72 °C was also given. The amplicons of colony PCR were resolved on 1.2 per cent agarose gel (Annexure III).

3.5.3.6 Sequencing of DNA clones

Once confirmed the presence of insert by colony PCR, the amplified products obtained from plasmid using T_7 and Sp_6 primers were directly send for sequencing. Sequencing was done at SciGenome, Regional centre, Kochi.

3.5.3.7 Maintenance of clones

Pure culture of recombinant bacteria was prepared. Single white colony from the transformed plate was taken by using flame sterilized bacterial loop. This was streaked on LBA plate containing ampicillin (50 mg l^{-1}). The plate was incubated overnight at 37°C and further stored at 4°C.

3.6 IN-SILICO ANALYSIS OF SEQUENCE

Sequence information of coat protein (CP) gene obtained was further analyzed using various bioinformatics tools for the characterization of the virus.

3.6.1 Homology search

The trimmed sequence was compared with sequence available in the National Centre for Biotechnological Information (NCBI) database using BLAST tool. Nucleotide-Nucleotide (blast-n) was carried out for the viral sequences to find the best aligned sequence using blastn tool available in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi ; Altschul *et al.*, 1997).

3.6.2 Detection of Open Reading Frame

Open reading frame (ORF) of the viral sequence was detected using ORF finder program available in NCBI (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). The nucleotide sequence was copied and pasted in the displayed box and selected 'orf find'. The displayed web page showed the ORF sequence in all reading frames. Open reading frame available in all the reading frames were noted and saved. The displayed nucleotide sequences of the reading frame were pasted in

notepad format and BLAST search was performed. The results were saved for further analysis and interpretation.

3.6.3 Amino acid analysis

The amino acid sequences were predicted using ExPASy translate tool (http://web.expasy.org/cgi-bin/translate). The properties of protein obtained from deduced amino acid sequence were determined by amino acid statistics tool (AASTASTS) available in Biology Workbench (http://seqtool.sdsc.edu/).

3.6.4 Phylogenetic Analysis

Phylogenetic analysis was conducted using MEGA 7.0 software by constructing Phylogenetic tree by Neighbor-Joining method (Saitou and Nei, 1987) and conducting boostrap analysis (1000 replicates). The CP gene sequences of BBrMV isolates generated in this study was aligned with 22 CP gene sequences of BBrMV isolates of banana and cardamom from India and Southeast Asia which were retrieved from NCBI (Table 7). Alignments of total 23 nucleotide sequences were done using Clustal Omega (http://www.ebi.ac.uk/ Tools/msa/clustalo) and the Phylogenetic relationship among BBrMV isolates from different geographical region was inferred using the Neighbor-Joining method.

Table 7: Details of selected coat protein sequences of BBrMV isolates used for Phylogenetic analysis.

SI. NO	Accession No	Isolate name	Host	Source	Reference
1	HQ709165	Card1	Cardamom	Madikere	Siljo *et al.*, 2012
2	HQ709166	Card2		Mudigere	
3	HQ709164	Card3		Sirsi	
4	HQ709163	Card5		Idukki	
5	HQ709162	Card6		Wynadu	
6	EU009210	TN4	Banana	Trichy	Selvarajan, R and Balasubramanian, V., unpublished data, 2014; unreferenced
7	KF385470	TN6	Banana	Pudukottai	Balasubramanian and Selvarajan, 2014
8	KF385472	TN8		Theni	
9	KF385474	TN10		Karur	
10	KF385476	TN12		Cuddalore	
11	KF385477	TN13		Tanjore	
12	KF385480	AP7		West Godhavari	
13	KF385481	KAR2		Bangalore	
14	KF385488	KER3		Kayankulam	
15	KF385490	KAR3		Arabhavi	
16	KF385491	KER2		Kasargod	
17	AY953427	AP1	Banana	Kovur	Ramesh, B., Sreenivasulu, P. and Krishna prasadji, J. unpublished data,2005; unreferenced
18	EU414267	P5	Banana	Philippines	Caruana, I.M.L., Bringaud, C. and Bousalem, M. unpublished data, 2008; unreferenced

Table 7 (Contd…)

19	AF071585	P2		Philippines	
20	AF071587	WS1	Banana	Western Samoa	Rodoni et al.,1999
21	AF071588	VT1		Vietnam	
22	AF071589	TH1		Thailand	

RESULTS

4. RESULTS

4.1 SYMPTOMATOLOGY

Symptomatology is very essential for early detection of disease. The banana plants infected with BBrMV showed different kinds of symptoms on different parts of the plant *viz.*, leaves, pseudostem, male-bud, and bunches. The primary symptoms of BBrMV infected plants exhibiting longitudinal irregular reddish streaks of varying sizes on the psuedostem of Nendran (AAB), Chenkadali (AAA) and Mysore poovan (AAB) (Plate1). The orientation of infected leaves became fan shaped which resembled travellers palm and such symptoms were noticed on banana var. Rasthali (AAB) (Plate 2A). Infected leaves showed spindle shaped lesions running parallel to the veins (Plate 2B) and mosaic pattern were visible on the lower side of petiole which extended throughout the midrib (Plate 2C). The symptoms seen on the bract were the main indication of disease in almost all the cultivars. The disease infected bracts showed a distinct reddish streaks and mosaic pattern (Plate 3A). The infected bunches were under sized with malformed fingers (Plate 3C) and mosaic pattern observed on peduncle (Plate 3B).

4.2 COLLECTION AND MAINTENANCE OF BBrMV CULTURE

Naturally infected suckers of variety Mysore poovan (AAB) showing the symptoms were collected from the fields of Banana Research Station, Kannara. The collected suckers were maintained under insect proof net house in the Department of Plant Pathology, College of Horticulture, Vellanikara (Plate 4B). Healthy tissue cultured plants were also maintained separately (Plate 4A). These were used for further study.

Plate 1: Symptoms on pseudostem showing linear red lesions on different cultivars: (A) Nendran (AAB) (B) Chenkadali (AAA) (C) Mysore poovan (AAB)

Plate 2: Symptoms on leaf: (A) Fan like arrangement of leaves (Variety Rasthali AAB) (B) Spindle shaped lesions running parallel to the veins on the leaf lamina (C) Mosaic on leaf petiole and midrib

Plate 3: Symptoms on Bract and fruits: (A) Reddish streaks and mosaic pattern on bract (B) Mosaic on peduncle (C) Mosaic symptoms on male bud (Bract) and undersized fruits of banana var. Chenkadali (AAB)

65
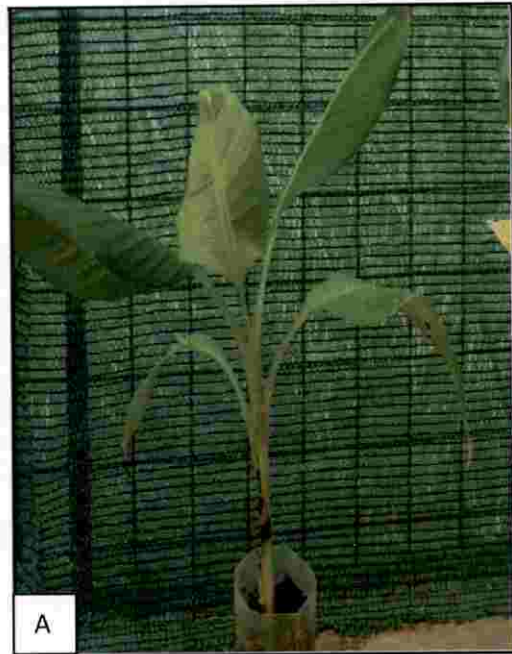
Plate 4: Maintenance of the BBrMV culture in insect proof net house Healthy (B) Infected

4.3 PROTEIN BASED DETECTION OF BBrMV

4.3.1 DAC-ELISA

4.3.1.1 Determination of titre of antibody

DAC-ELISA was validated for determining the antibody titre with different dilutions of (1:100; 1:200; 1:300; 1:500) antiserum. The absorbance value was recorded using a VESAMAX Microtitre plate reader. The results obtained 30 minutes after the addition of substrate are presented in Table 8 and Plate 5. In the present investigation, it was found that BBrMV could be detected in the primary antibody dilution ranging from 1:200 to 1:500 along with 1:500 dilution of alkaline-phosphatase conjugated secondary antibody. However, maximum colour development was observed at 1: 200 dilution.

Table 8: Determination of antibody titre

Sample	Absorbance at 405 nm					
	Control	Healthy	1: 100	1:200	1:300	1:500
1	0.105	0.167	0.580	0.610	0.573	0.425
2	0.119	0.178	0.570	0.580	0.589	0.455
3	0.130	0.160	0.560	0.650	0.560	0.445
Average	0.118	0.168	0.570	0.613	0.574	0.441

4.3.1.2 Detection of BBrMV by DAC-ELISA

Antibody purchased from Agdida, USA and samples from both suspected and healthy leaf samples of banana plant were used to perform DAC-ELISA. A titre of antiserum used for the study was 1:200 which was previously determined by DAC-ELISA in section 4.3.1.1.The absorbance value at 405 nm VERSAMAX ELISA reader was recorded. The result of the experiment revealed that the BBrMV specific antiserum gave higher reactivity and it could give clear difference between healthy and infected sample (Plate 6; Table 9). The average absorbance value of infected samples was almost thrice as that of healthy samples.
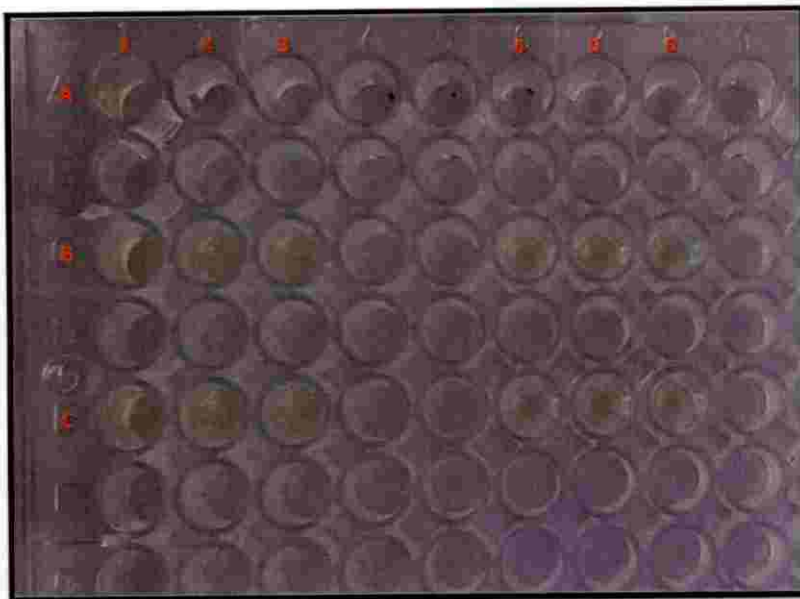
Plate 5: Determination of antibody titre: Control: A (1-3); Healthy: A (4-6); Infected: 1:100-B (1-3), 1:200-C (1-3), 1:300-B (4-5), 1:500- C(4-5)
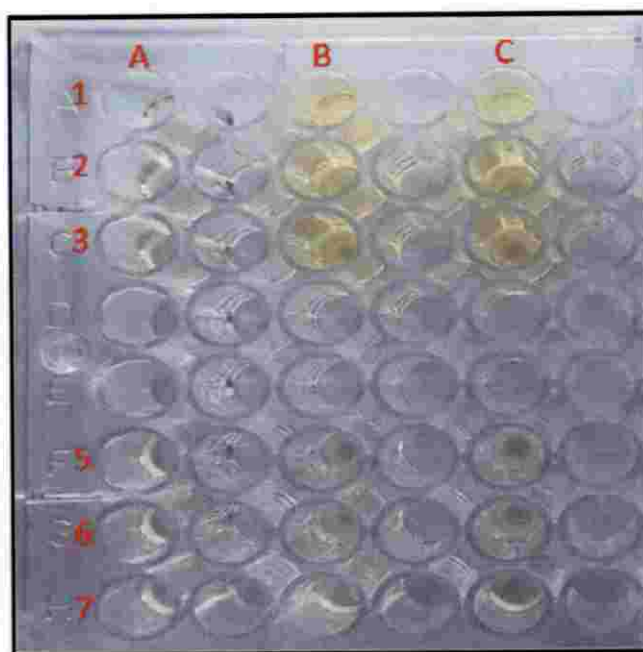
Plate 6: Detection of BBrMV by DAC-ELISA: Control: A (1-7); Healthy: B (5-7) C (5-7); Infected B (1-3) C (1-3).

Table 9: Absorbance value of DAC-ELISA at 405 nm

Sample	Sample 1	Average	Sample 2	Average
Control	0.159		0.198	
	0.148	0.137	0.131	0.167
	0.104		0.173	
Healthy	0.292		0.207	
	0.286	0.267	0.236	0.244
	0.223		0.293	
Infected	0.737		0.978	
	0.640	0.696	0.830	0.915
	0.712		0.937	

4.3.2 Detection of BBrMV by DIBA

DIBA is a simple serological method of detection, widely followed for detection of a virus. Antigen isolated in a crude protein fraction from an infected leaf sample was coated onto a nitrocellulose membrane and virus was detected with specific antibody. DIBA was conducted to detect the presence of virus in suspected and healthy sample using monoclonal antibody specific to BBrMV. The result of the experiment was assessed by visual observation by comparing the intensity of the colour. The infected leaf sample showed the purple coloured spots on nitrocellulose membrane indicating positive reaction which is absent in both healthy samples and the buffer check (Plate 7).

4.4 NUCLEIC ACID BASED DETECTION OF BBrMV INFECTION

4.4.1 Isolation of total RNA

The leaf samples from infected and healthy banana plants maintained in net house were taken for total RNA isolation. There was a difference in the quality and quantity of total RNA isolated by different protocols. .

Although the total RNA was isolated from leaf samples using PureLink ® Plant RNA Reagent and TRIzol reagent, the protocol with PureLink ® Reagent
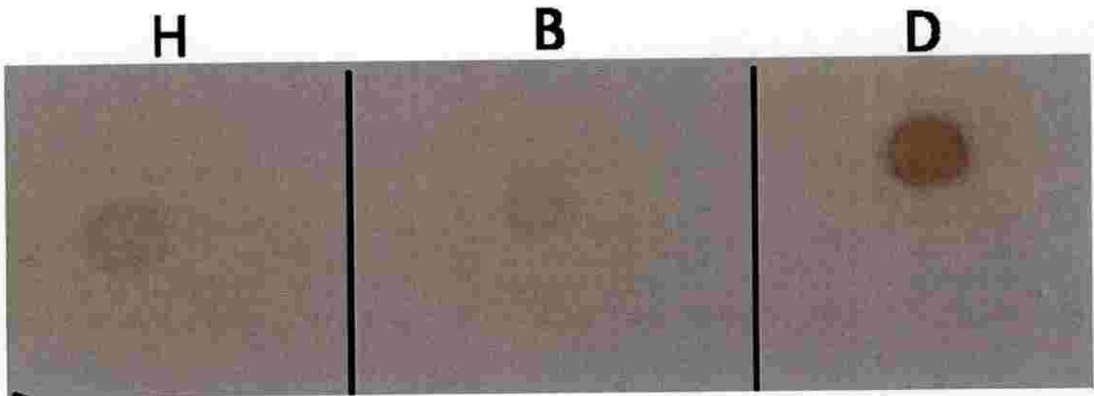
Plate 7: Detection of virus by Dot immuno binding assay (DIBA)

       H- Healthy sample

       B- Buffer control

       D- Infected sample

provided higher quality and quantity of RNA (Table 10). The total RNA isolated was resolved on agarose gel (1.5%) and both methods yielded two distinct rRNA bands but PureLink ® Reagent method showed thick bands compared to TRIzol reagent and there was no apparent RNA degradation in both the methods (Plate 8 and 7). Among the two different methods evaluated for RNA isolation from banana leaves, PureLink ® Reagent method provided highest quality and quantity of the RNA as revealed by spectrophotometer analysis and gel electrophoresis.

Table 10: Yield and absorbance ratio of total RNA isolated from leaf samples by different protocols

Method	Sample	RNA yield (ng/ ul)	Absorbance value $A_{260/280}$
TRIzol method	1	1908.0	1.72
	2	1152.1	1.67
	3	1210.3	1.78
PureLink ® Plant RNA Reagent method	1	2002.8	2.05
	2	1354.7	2.00
	3	1223.2	2.11
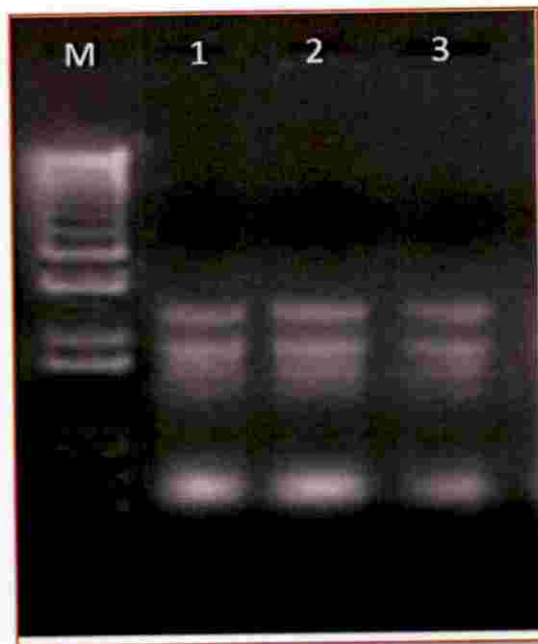
Plate 8: Total RNA isolated from banana leaf using TRIzol reagent: M-Marker (1 kb plus); Lane 1- Healthy control ; Lane 2 & 3- Infected.

Plate 9 : Total RNA isolated from banana leaf using PureLink ® Plant RNA Reagent: M- Marker (1 kb plus); Lane 1- Healthy control; Lane 2 & 3-Infected.

4.4.2 RT-PCR amplification of partial coat protein gene of BBrMV

The first strand cDNA was synthesized by using RevertAid H Minus First Strand cDNA synthesis kit (Thermo Scientific). The annealing temperature of B1 and B2 primers were standardized as 58.2°C using gradient PCR (Plate 10).

RT-PCR analysis with reported primer set, B1 and B2 yielded amplicons of ~600 bp size in samples positive for virus infection (Plate 11). The amplicons of positive samples were eluted from gel for obtaining purified product. Reamplification of RT-PCR products as well as gel elutes produced amplicons of expected band size confirming the presence of virus infection and which was further used for cloning.

4.4.3 DNA sequencing

Partial coat protein gene of size 560 bp including C terminus of the CP and the 3^1 untranslated region of the BBrMV genome obtained by sequencing the cloned RT-PCR product (~605 bp size) is given Fig. 4.

4.4.4 Sequence analysis

Blast analysis of cloned product confirmed the presence of virus. Nucleotide BLAST (Blastn) analysis was carried out using trimmed sequence and result revealed that it was more similar to BBrMV-Coimbatore isolates (Accesion No-AY494979) with 98 per cent identity. Sequence was also shown maximum homology of 98 per cent to other three accession namely, AF071582 (isolate I1), EU531470 (Coimbatore isolate) AY529121 (Tiruchirapalli) with minimum E value (Fig. 5b). There was 72 blast hits against present sequence with 97 per cent query coverage (Fig. 5a).

1	48.2 °C
2	48.5 °C
3	49.6 °C
4	51.6 °C
5	52.2 °C
6	53.4 °C
7	54.6 °C
8	55.6 °C
9	56.9 °C
10	58.2 °C
11	59.6 °C
12	60.0 °C
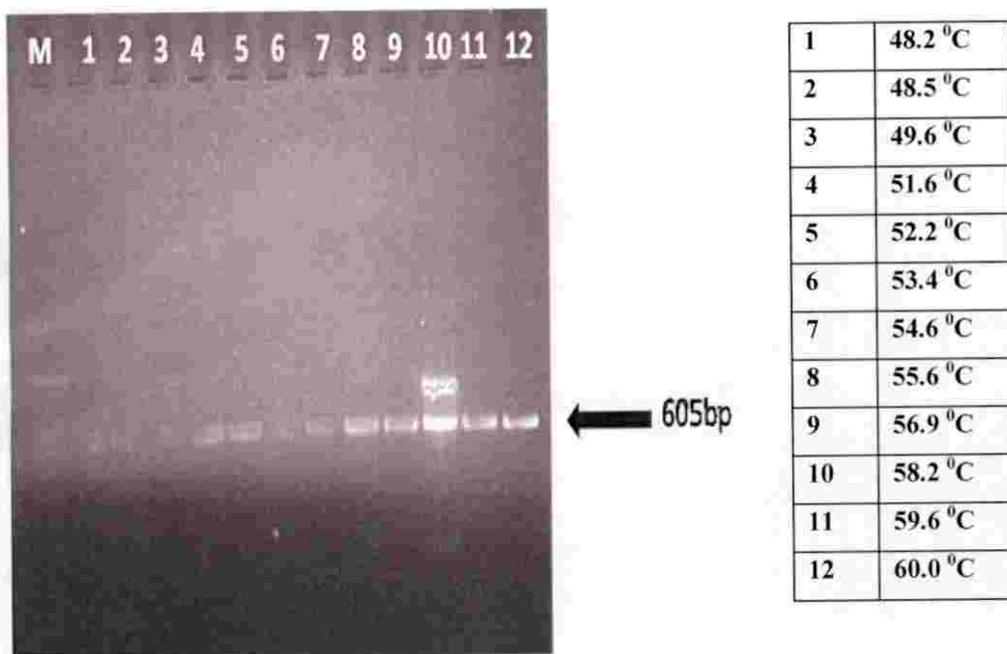
Plate 10: Standardization of annealing temperature for B1and B2 primers using gradient PCR: M: Marker (100bp plus); Lane 1 – 12: 48^{0}C - 60^{0}C.
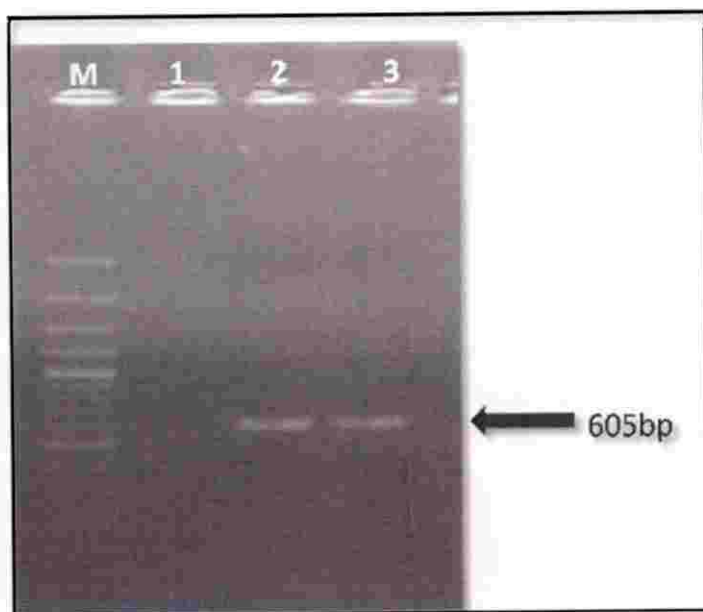
Plate 11: Detection of BBrMV by RT-PCR using B1 and B2 primers: M- Marker (100bp plus); Lane 1 – Healthy control; Lane 2&3-Infected samples showing amplified product of 605bp.

5'GGCACATGGAGTATGATGGATAGGGGTGAGCAATTAGTTTACC
AGTTAAAGCCTATTATTGAGAACGCTCAGCCTACTTTTCGGCAAA
TTATGGCACATTTTTCTGATGCTGCTGAGGCATACATAACAATGC
GCAATGTCACGGAGAGATATATGCCTAGGTGGGGAGCACTTAGG
GGATTGAATGACATAAGCTTAGCCCGATATGCATTTGATTTTTAC
GTAGTCACATCAAAAACTACAAACAGGGCTAGAGAAGCACACAC
GCAGATGAAAGCTGCAGCTATTCGTGGGTCAAACACTCGGTTATT
CGGTTTGGATGGAAATCTTGGACCTGGTAAGAGAATACAGAGAG
GCATACCGTTGAAGATGTGAAGCGTGATATGCACTCTCTGCTTGG
GATGAAACATGAATAGATAAATAGTTATCTGGAGCTTGCTCCCTA
TAATTATGTGTGCTTTATGTTATTGTGATAATTGTAGTGTGAGCTT
CTCACCTAAGTACCTACATGTATTGTGTGTGGTATTTATATATTCA
CAATACGCAAAGGGACCGCCTGT 3'

Figure 4: Sequence of CP gene amplified by B1/B2 primer

Distribution of 72 Blast Hits on the Query Sequence

over to show defline and scores, click to show alignments

Color key for alignment scores

| <40 | 40-50 | 50-80 | 80-200 | >=200 |

Query
1   100   200   300   400   500
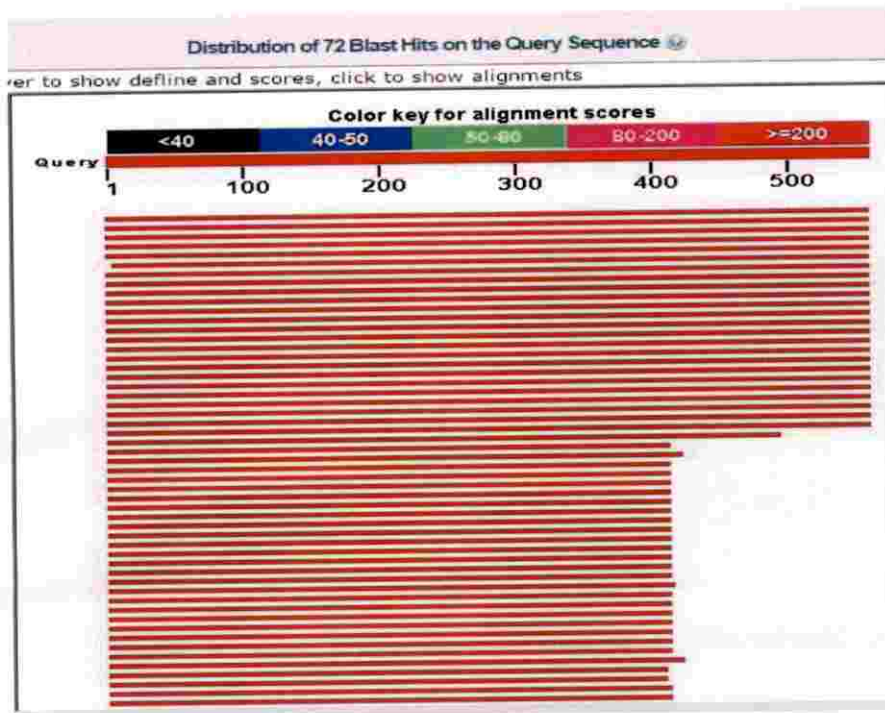
Figure 5a: Blastn graphical output of partial coat protein region and 3'UTR region of BBrMV

Sequences producing significant alignments:

Select: All None Selected 0

Description	Max score	Total score	Query cover	E value	Ident	Accession
Banana bract mosaic virus isolate Coimbatore chlsignatus mRNA, partial cds	974	974	100%	0.0	98%	AYA94979.1
Banana bract mosaic virus isolate P1 polyprotein mRNA, partial cds	974	974	100%	0.0	98%	AFG71062.1
Banana bract mosaic virus isolate Coimbatore coat protein gene, partial cds	963	963	100%	0.0	98%	EU634473.1
Banana bract mosaic virus clone stemvirus ADA coat protein gene, partial cds, and 3 UTR	963	963	100%	0.0	98%	AY528123.1
Banana bract mosaic virus CdhI from India polyprotein gene, partial cds	962	962	100%	0.0	97%	AH775027.1
Banana bract mosaic virus isolate D polyprotein mRNA, partial cds	939	939	99%	0.0	97%	AFG71063.1
Banana bract mosaic virus isolate TRY, complete genome	935	935	100%	0.0	97%	HM131474.1
banana bract mosaic virus, complete genome	924	924	100%	0.0	96%	DQ851488.1
Banana bract mosaic virus isolate Tenaspnam coat protein gene, partial cds	918	918	100%	0.0	96%	JN915968.1
Banana bract mosaic virus from India polyprotein gene, partial cds	917	917	100%	0.0	96%	EU659732.1
Banana bract mosaic virus isolate P1 polyprotein mRNA, partial cds	917	917	100%	0.0	96%	AFG71067.1
Banana bract mosaic virus isolate Niresunu coat protein gene, partial cds	913	913	100%	0.0	96%	JN915958.1
Banana bract mosaic virus from Philippines polyprotein gene, partial cds	913	913	100%	0.0	96%	EU414067.1
Banana bract mosaic virus isolate Niccro coat protein gene, partial cds	907	907	100%	0.0	96%	JN916071.1
Banana bract mosaic virus isolate Akudo coat protein gene, partial cds	907	907	100%	0.0	96%	JN915957.1
Banana bract mosaic virus isolate BBr07 nt coat, complete genome	896	896	100%	0.0	96%	KT426631.1
Banana bract mosaic virus isolate Sitra coat protein gene, partial cds	896	896	100%	0.0	96%	JN915976.1

Figure 5b: Blastn text output of partial coat protein region and 3'UTR region of BBrMV

4.5 AMPLIFICATION OF COAT PTOTEIN (CP) REGION OF VIRUS

4.5.1 Primer designing

The forward (F) and reverse (R) primers were designed for the CP gene of BBrMV based on the most favorable combination of conserved regions in the multiple aligned nucleotide sequences and named as BCPFl and BCPRl respectively.

BCPFl sequence: 5' GATGATGACCCAAGCCGC 3'

BCPRl sequence: 5' GCAGAGAGT GCATATCAC 3'

4.5.1.1 Primer evaluation

The validation of primers was done using OligoAnalyzer 3.1 program revealed that it holds good ideal primer parameters such as GC content and annealing temperature and also the designed primer did not showed any hairpin formation and self dimer. The annealing temperature was standardized as 58^0C by gradient PCR (Plate 12).

4.4.2 Uncoupled RT-PCR

The primer pair BCPFl/Rl amplified a product of ~ 850 size (Plate 13). The positive samples showing expected amplicons of 850 bp were eluted and re-amplified which was used for cloning.

4.5.3 Cloning and sequencing

The re-amplified CP gene of BBrMV were cloned into vector plasmid pGEM-T and transformed into *E. coli* strain DH5α. The transformed colonies were selected based on blue/white selection (Plate 14) and further confirmation was done by colony PCR (Plate 15).The amplified product of colony PCR was used for sequencing and sequence of CP gene (788 bp) was obtained from automated sequencing using T7 and SP6 primer pair is given in Fig. 6.
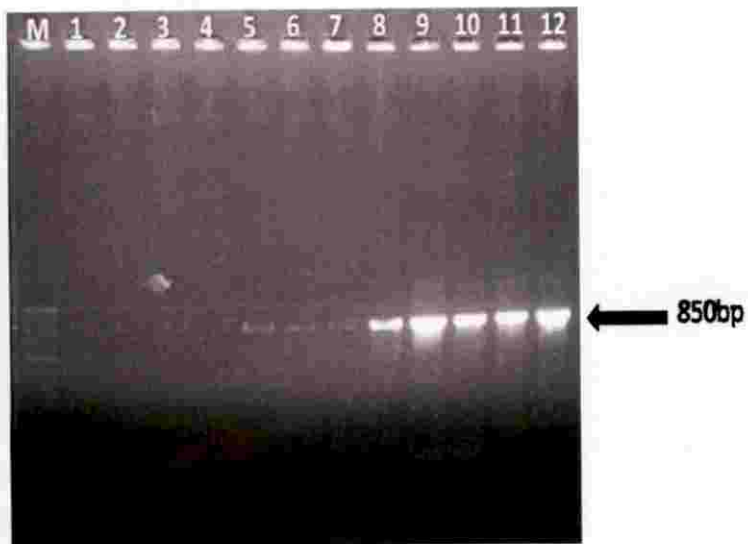
1	48.2 ^{0}C
2	48.5 ^{0}C
3	49.6 ^{0}C
4	51.6 ^{0}C
5	52.2 ^{0}C
6	53.4 ^{0}C
7	54.6 ^{0}C
8	55.6 ^{0}C
9	56.9 ^{0}C
10	58.2 ^{0}C
11	59.6 ^{0}C
12	60.0 ^{0}C

Plate 12: Standardization of annealing temperature for BCF1 and BCR1 primer using gradient PCR: M: Marker (100bp plus); Lane 1 – 12: 48^{0}C -60^{0}C.
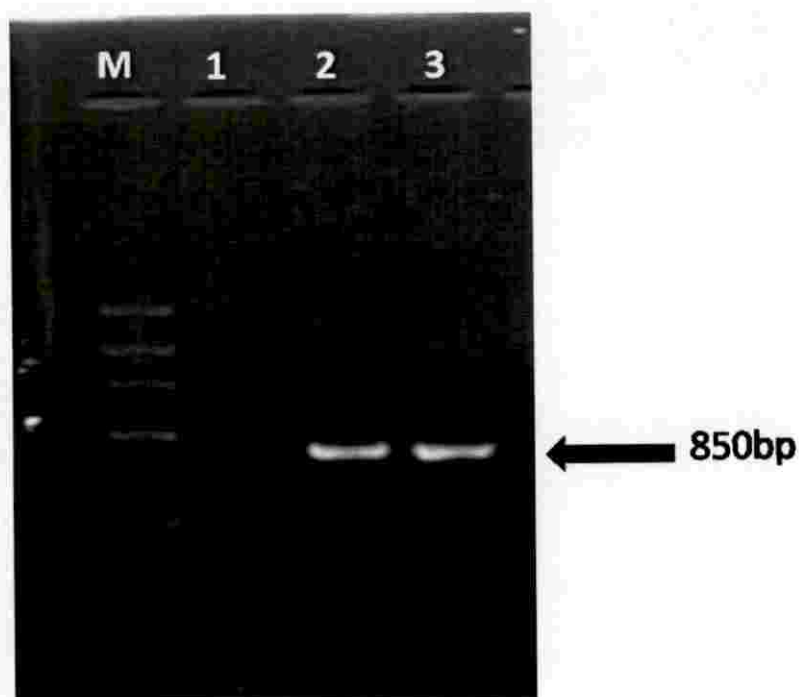
Plate 13: Amplification of CP region by BCF1/R1: M- Marker (100bp plus); Lane 1 -Healthy control; Lane 2&3- Infected samples.
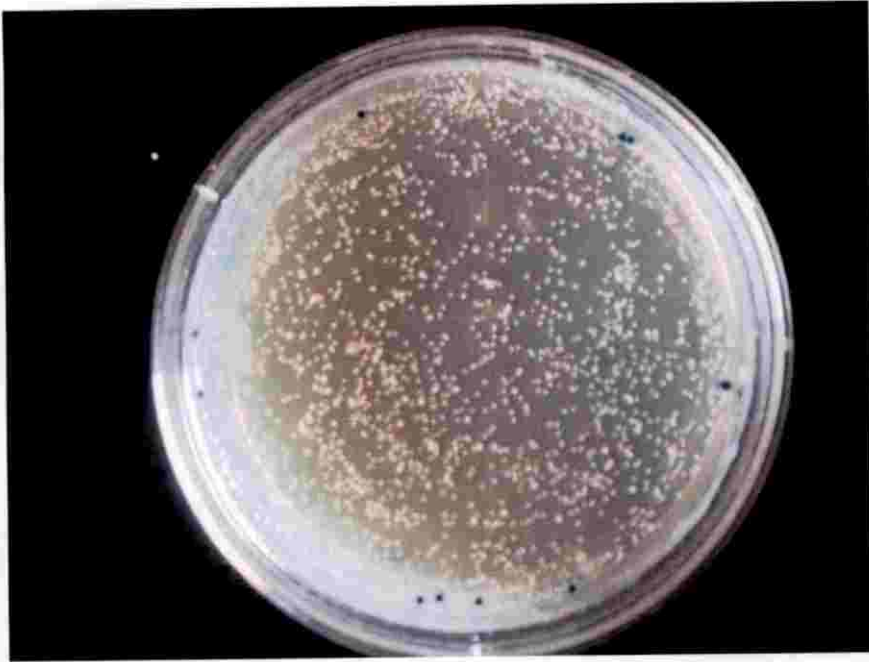
Plate 14: Transformation *of E. coli* **(DH5 alpha) cells with CP gene of BBrMV (White colonies- Transformed, blue colonies- Untransformed)**

1150bp
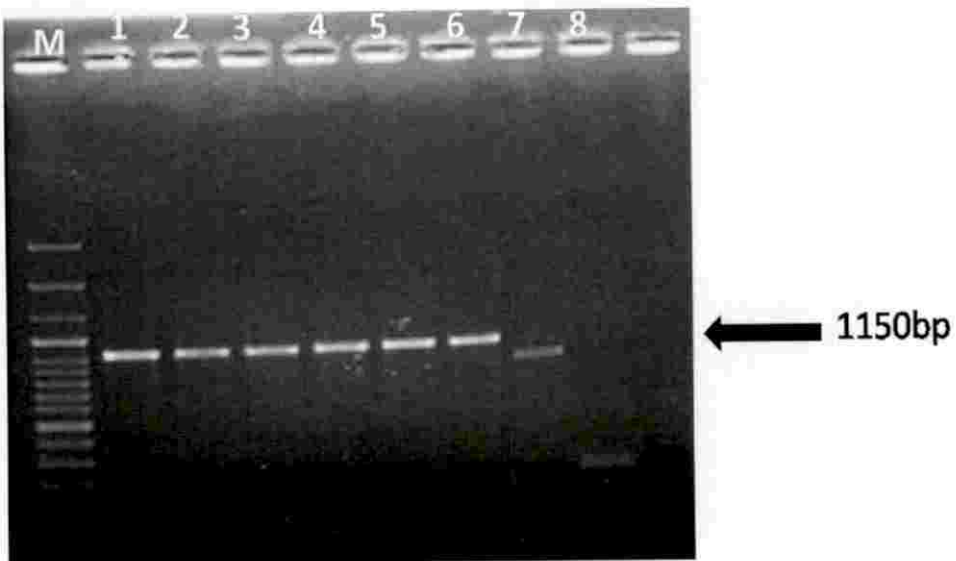
Plate 15: Analysis of recombinant clones using colony PCR: M- Marker (100bp plus); Lane 1 to 6- Recombinant plasmid; Lane7-Positive control (DNA insert); Lane 8-Negative control (Non recombinant plasmid).

5'AACCACTACAACAGCTCCAAGCACATTTGGACAACCCACGACTA
CGTCAGCTCCATCTTCATCAAGCACGCCACCCAGAGCTTCCACGCA
AATTGCACCAGTTCGGGATCGTGACGTTGATGCTGGTAGTACAAA
CTTTATCATCCCTCGAATTAAACCGATGACTGGGAAAATGCGTCTC
CAAGGTATCGAGGAAAAACTGCAATTAATGTCGAGTTCTTGCTTC
AATATAAGCCCGATCAGTTTGATTTGTCAAATGCTATCGCAACTAG
GGAGCAATATGATGCGTGGTGTGATGCTGTGAAACGTGAGTATGC
CATAGAGGACGAAGAACAGTTTACAACCTTGTTAGGTGGTTTGAT
GGTATGGTGCATAGAGAATGGGACATCACCAAATTTGAATGGTAC
ATGGAGTATGATGGATAGGGGTGAGCAATTAGTTTACCAGTTAAA
GCCTATTATTGAGAACGCTCAGCCTACTTTTCGGCAAATTATGGCA
CATTTTTCTGATGCTGCTGAGGCATACATAACAATGCGCAATGTCA
CGGAGAGATATATGCCTAGGTGGGGAGCACTTAGGGGATTGAATG
ACATAAGCTTAGCCCGATATGCATTTGATTTTTACGTAGTCACATC
AAAAACTACAAACAGGGCTAGAGAAGCACACACGCAGATGAAAG
CTGCAGCTATTCGTGGGTCAAACACTCGGTTATTCGGTTTGGATGG
AAATCTTGGACCTGGTGAAGAGAATACAGAGAGGCATACCGTTGA
AGATGTGAAGCGTGATAT 3'

Figure 6: Sequence of CP gene (788 bp) amplified by BCPF1/R1 primer

4.5.4 Sequence analysis

Nucleotide Blast analysis (blastn) of full coat protein region showed significant homology with reported coat protein gene sequences in NCBI data bank. The sequence had shown the maximum homology of 99 per cent to accession KF385491 (Kerala isolate) with 100 per cent query coverage and there were 74 blast hits reported for sequence (Fig. 7a and 7b). Deduced amino acid sequence obtained by ExPASy translate tool is given Fig. 8. Deduced amino acid of length 136 exhibited 95 percent to 83 percent identity (Fig. 9) with other BBrMV protein sequence in database.

The sequence were translated into five possible open reading frame (ORF) (Fig. 10), with longest one in +1 frame starting from base 361 to 786 having length of 426 bp with 141 amino acid. The phylogenetic trees were constructed from the alignment of CP gene sequences of 23 isolates (Fig. 11) and revealed that the present isolate was more similar to KER2 isolate (KF385491) from Kasargud (99% nucleotide sequence similarity) which is clustering with TN13. BBrMV isolates were grouped into two distinct major groups based on genetic distances. All Indian isolate clustered in group I and the remaining Southeast Asian isolates (Philippines, Thailand, Vietnam, and Western Samoa) were together in group II. Within group I, all cardamom isolates card2, card5, card6, card3 except card1 were clustered together. Isolates of Andra Pradesh (AP1, AP7), Karnataka (KAR2, KAR3), Tamil Nadu (TN8, TN12) were separately clustered within the group I whereas other isolates (TN4, TN6, TN10, TN13, KER2, KER3) including isolate generated in this study (BCP1) were diverged irrespective of geographical distribution.
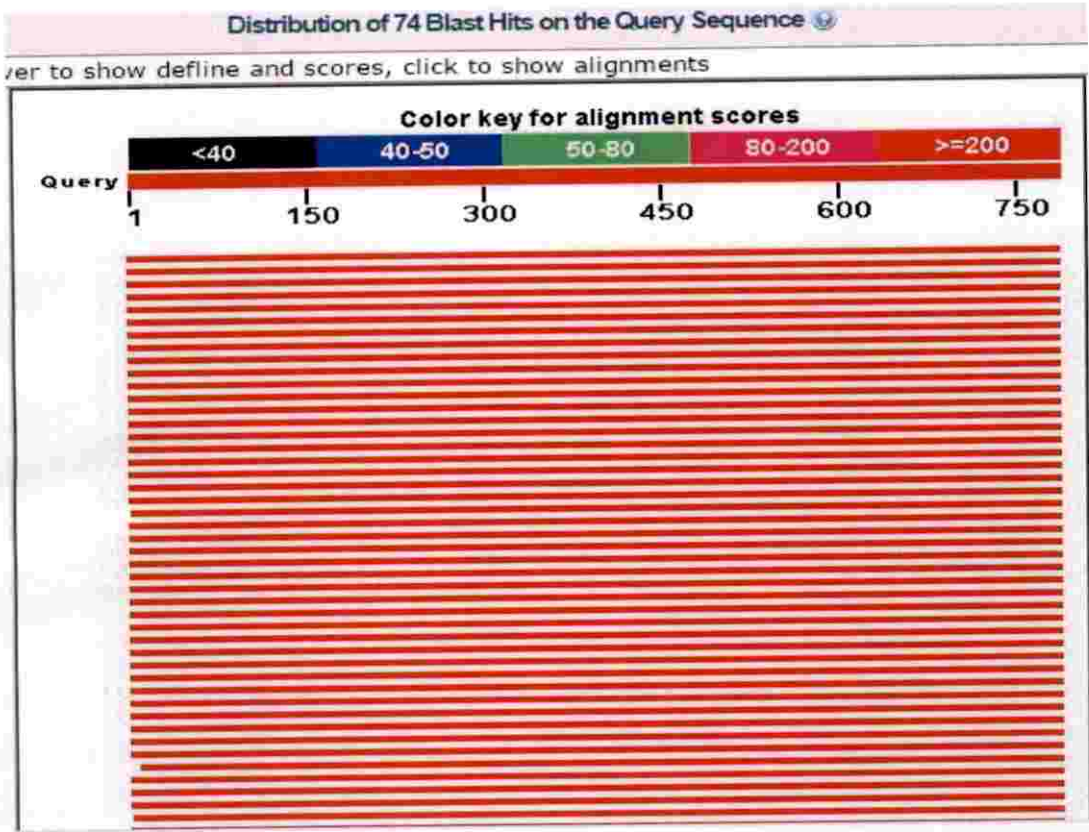
Figure 7a: Blastn graphical output of CP sequence region of BBrMV

Sequences producing significant alignments:

Select: All None Selected 0

Description	Max score	Total score	Query cover	E value	Ident	Accession
Banana bract mosaic virus isolate KER 2 coat protein gene, partial cds	1395	1395	100%	0.0	99%	KF385491.1
Banana bract mosaic virus isolate TN17 coat protein gene, partial cds	1384	1384	100%	0.0	98%	KF385485.1
Banana bract mosaic virus isolate I1 polyprotein mRNA, partial cds	1384	1384	100%	0.0	98%	AF071582.1
Banana bract mosaic virus isolate TN9 coat protein gene, partial cds	1378	1378	100%	0.0	98%	KF385473.1
Banana bract mosaic virus isolate Quintabala polyprotein mRNA, partial cds	1378	1378	100%	0.0	98%	AY494979.1
Banana bract mosaic virus isolate TN8 coat protein gene, partial cds	1373	1373	100%	0.0	98%	KF385472.1
Banana bract mosaic virus isolate Card-3 coat protein gene, partial cds	1373	1373	100%	0.0	98%	HG709164.1
Banana bract mosaic virus isolate Card-6 coat protein gene, partial cds	1373	1373	100%	0.0	98%	HG709162.1
Banana bract mosaic virus isolate TN13 coat protein gene, partial cds	1367	1367	100%	0.0	98%	KF385477.1
Banana bract mosaic virus isolate Kamataka polyprotein gene, partial cds	1367	1367	100%	0.0	98%	EF654655.1
Banana bract mosaic virus isolate TN10 coat protein gene, partial cds	1362	1362	100%	0.0	98%	KF385474.1
Banana bract mosaic virus isolate Card-1 coat protein gene, partial cds	1362	1362	100%	0.0	98%	HG709165.1
Banana bract mosaic virus isolate Trichy coat protein (cp) gene, partial cds	1362	1362	100%	0.0	98%	EU009210.1
Banana bract mosaic virus isolate Card-5 coat protein gene, partial cds	1356	1356	100%	0.0	98%	HG709163.1
Banana bract mosaic virus isolate TN12 coat protein gene, partial cds	1351	1351	100%	0.0	98%	KF385476.1

Figure 7b: Blastn text output of CP sequence region of BBrMV

VWCIENGTSPNLNGTWSDRGEQLVYQLKPIIENA
QPTFRQIAHFSDAAEAYIT RNVTERYPRWGALRG
LNDISLARYAFDFYVVTSKTTNRAREAHTQ KAA
AIRGSNTRLFGLDGNLGPGEENTERHTVEDVKRD

Figure 8: Deduced amino acid sequence of CP gene obtained ExPASy translate tool

Distribution of 100 Blast Hits on the Query Sequence

Mouse-over to show defline and scores, click to show alignments

Color key for alignment scores

| <40 | 40-50 | 50-80 | 80-200 | >=200 |
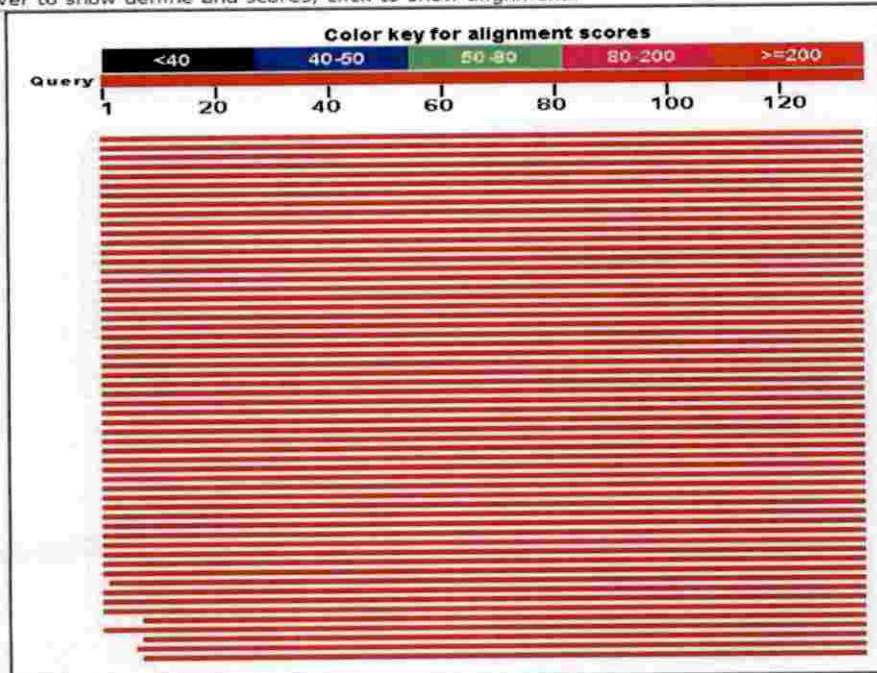
a. **Graphical output of blastp**

Sequences producing significant alignments:

Select: All None Selected 0

Description	Max score	Total score	Query cover	E value	Ident	Accession
coat protein [Banana bract mosaic virus]	260	260	100%	2e-86	95%	AIR08510.1
coat protein [Banana bract mosaic virus]	260	260	100%	3e-86	95%	AIR08585.1
coat protein [Banana bract mosaic virus]	252	252	100%	2e-86	95%	AIC33453.1
polyprotein [Banana bract mosaic virus]	252	252	100%	2e-86	95%	AAC25498.1
CP protein [Banana bract mosaic virus]	252	252	100%	2e-86	95%	YP_001718531.1
coat protein [Banana bract mosaic virus]	252	252	100%	2e-86	95%	ABK32248.1
polyprotein [Banana bract mosaic virus]	252	252	100%	2e-86	95%	AAC25411.1
polyprotein [Banana bract mosaic virus]	252	252	100%	2e-86	95%	AAC25498.1
coat protein [Banana bract mosaic virus]	251	251	100%	2e-86	95%	AHZ74064.1
polyprotein [Banana bract mosaic virus]	252	252	100%	2e-86	95%	AAC25494.1
coat protein [Banana bract mosaic virus]	252	252	100%	2e-86	95%	AER42634.1
coat protein [Banana bract mosaic virus]	252	252	100%	3e-86	95%	AIC33459.1
coat protein [Banana bract mosaic virus]	252	252	100%	3e-86	95%	ADN84919.1
coat protein [Banana bract mosaic virus]	252	252	100%	3e-86	95%	AIC33455.1
coat protein [Banana bract mosaic virus]	252	252	100%	3e-86	95%	AIC33456.1
coat protein [Banana bract mosaic virus]	252	252	100%	3e-86	95%	AHB65163.1
coat protein [Banana bract mosaic virus]	252	252	100%	3e-86	95%	AIC33454.1
coat protein [Banana bract mosaic virus]	252	252	100%	3e-86	95%	AIC33474.1
coat protein [Banana bract mosaic virus]	252	252	100%	3e-86	94%	AER42632.1

b. **Text output of blastp**

Figure 9: Blastp analysis of deduced amino acid sequence of CP region of BBrMV

Open Reading Frame Viewer

Contig - Desktop

ORFs found: 5   Genetic code: 1   Start codon: 'ATG' only
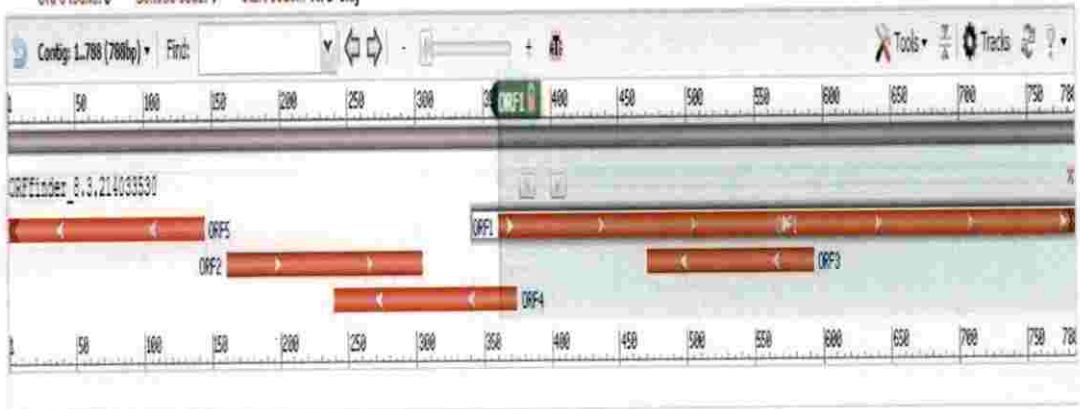
Label	Strand	Frame	Start	Stop	Length (bp | aa)
ORF1	+	1	361	>786	426 | 141
ORF2	+	2	161	304	144 | 47
ORF5	-	3	144	>1	144 | 47
ORF4	-	1	374	240	135 | 44
ORF3	-	1	593	471	123 | 40

Figure 10: Open Reading Frame (ORF) of CP region of BBrMV
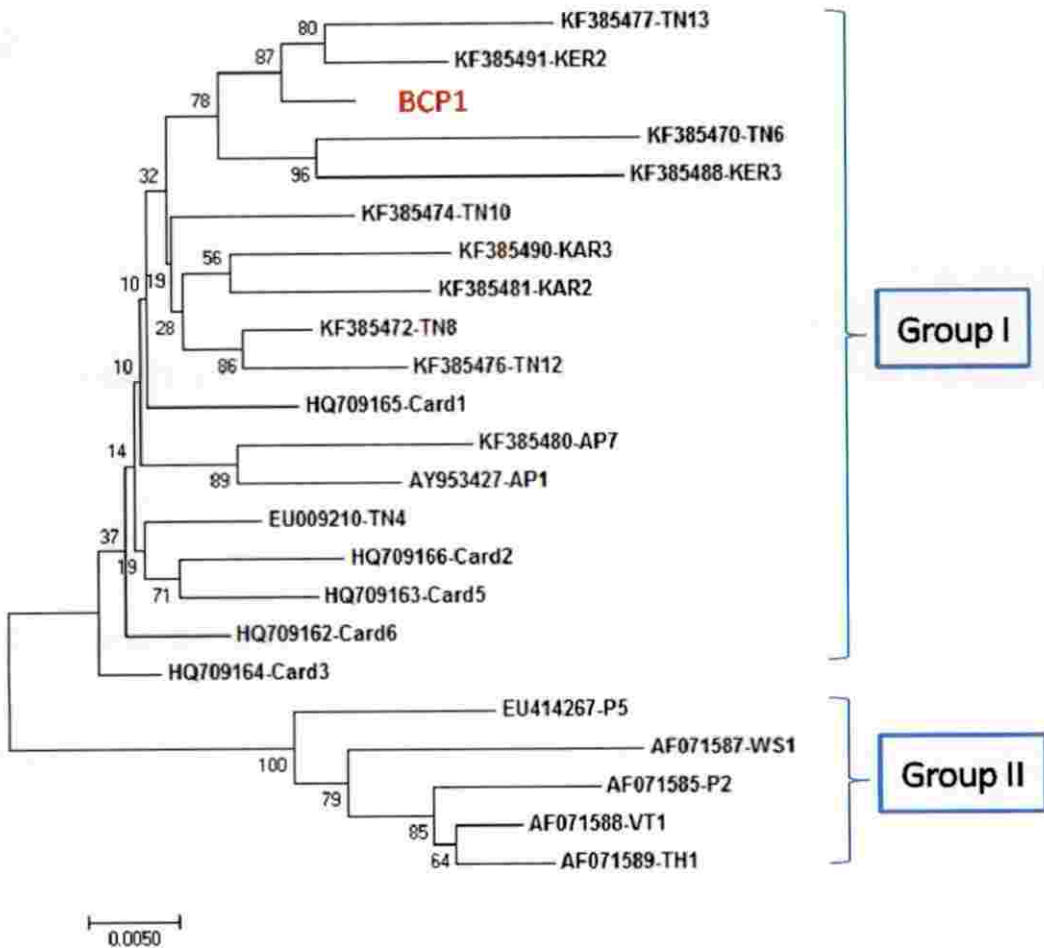
Figure 11: Phylogenetic analysis of coat protein gene of *Banana bract mosaic virus* Isolates. Tree was constructed by Neighbor-joining method using mega 7. Numbers are the percentage support of branching based on bootstrap analysis (1000 replications). Scale bar indicates 0.05 substitution/ site.

DISCUSSION

5. DISCUSSION

Banana and plantains are the important tropical fruit crops which are grown as staple food in many developing countries. It has high starch and protein content which contribute to food security of millions of people as well as provides income through local and international trade (Aurore *et al.*, 2009). Even though banana is widely distributed globally, the high susceptibility to diseases is a major limitation to its profitable and sustainable production. Diseases especially those caused by viruses are major threat for its cultivation because of the abundance of insect vectors and presence of many alternate hosts (Magnaye and Valmayor, 1995). Vegetative propagation, insect transmission and uncontrolled introduction of infected banana germplasm have contributed to the widespread distribution of viral infection in this crop. Plant viruses cause some of the world's leading plant diseases, leading to the loss of billions of dollars annually and at the same time viruses are one of the most difficult group of plant pathogens for detection and management.

There are four major viruses causing disease in banana *viz., Banana bract mosaic virus* (BBrMV), *Banana bunchy top virus* (BBTV), *Banana streak virus* (BSV) and *Cucumber mosaic virus* (CMV) (Diekmann and Putter, 1996). Banana bract mosaic is one of the most important viral diseases, which leads to an yield reduction ranging from 52 to 75% (Cherian *et al.*, 2002). The disease is caused by *Banana bract mosaic virus* (BBrMV) which is a member of genus *Potyvirus* and family *Potyviridae*.

In the case of viral diseases like banana bract mosaic, early detection and diagnosis of infection is very important since symptomless hosts might carry the viral inoculum. Since banana is a vegetatively propagated plant, there is more chance of carrying latent infection from the infected mother plants. Disease diagnosis and early detection of the pathogen residing in the planting materials could be done through various molecular and serodiagnostic indexing techniques which have been developed and validated by various virus indexing laboratories.

Still more sensitive assays need to be developed. Good quality antiserum is a pre-requisite for various serodiagnostic techniques like ELISA, DIBA etc. But, the availability of high titre antiserum is the difficulty faced by the virus Indexing laboratories. BBrMV infected banana are poorly characterized, which is a hindrance for the safe movement of germplasm.

Hence in the present study, attempts were made to develop the molecular clones of coat protein (CP) gene of BBrMV and to characterize it. This would help to develop recombinant clones which have immense application in the field of disease diagnostics and management. These clones could be used for the production of antiserum through recombinant DNA technology and for coat protein mediated resistance through genetic engineering. These could also be used as disease diagnostic probes for more sensitive molecular techniques like Nucleic acid spot hybridization. As CP gene sequences are frequently used to develop pathogen derived resistance against viral infection by means of genetic engineering, Results of the present study on characterization of CP gene could help in predicting the risk of breakdown of resistance, when BBrMV resistant transgenic banana lines are developed for the management of disease and for safe germplasm exchange.

Banana bract mosaic disease was first reported as kokkan disease of unknown etiology in Kerala by (Samraj et al., 1996). Later, Singh et al.2000 had authentically reported that the disease on banana cv. Nendran was caused by BBrMV. Presently, BBrMV is widely spread in banana growing regions of India viz., Kerala, Tamil Nadu, Karnataka and Andhra Pradesh (Singh et al., 2000; Cherian et al.,2002 and Kiranmai et al., 2005). The yield loss due to this disease in Kerala was 70 and 40 per cent was recorded on banana cvs. Robusta and Nendran respectively (Cherian et al., 2002).

BBrMV was transmitted in a non-persistent manner by several aphid including *Rhopalosiphum maidis* and *Aphis gossypii* (Magnaye and Espino 1990), *Pentalonia nigronervosa Cocq* (Bateson and Dale 1995; Thomas and Magnaye,

1996).The disease also spreads through vegetative planting materials such as suckers and tissue cultured plantlets but it was not soil-borne (Thomas and Magnaye, 1996).

5.1 SYMPTOMATOLOGY

In the present study, the various symptoms associated with *Banana bract mosaic virus* (BBrMV) on the different parts of the banana *viz.*, leaves, pseudostem, male bud, and bunches were monitored and documented under natural field conditions. Plants infected with BBrMV showed various symptoms *viz.*, longitudinal, irregular, reddish streaks of varying length on the base of pseudostem, mosaic pattern on the bracts, fan like arrangement of leaves (resembled like a travellers palm), spindle shaped lesions and streaks on leaf lamina and malformed and under sized fruit were observed under field condition. These kind of symptoms were reported earlier by many workers across the world (Magnaye and Espino, 1990; Bateson and Dale, 1995; Magnaye and Valmayor, 1995; Thomas and Magnaye, 1996; Samraj *et al.*,1996; Balakrishnan *et al.*, 1996; Singh *et al.*, 1996; Rodoni *et al.*, 1997; Dhanya, 2004).

5.2 PROTEIN BASED DETECTION OF BBrMV

Diagnostics is important in the development and implementation of disease management strategies. These tools could be categorized into three main groups; biological characterization using symptomatology, serological detection using specific antigen-antibody recognition and molecular characterization based on virus nucleic acids.

In the present study, serological methods *viz.*, DAC-ELISA, DIBA and Nucleic acid based method (RT-PCR) were validated to detect BBrMV in the plant samples. Out of the three techniques employed, DAS-ELISA and DIBA were protein-based and relied entirely on the use of antiserum. But the RT-PCR is entirely based on the nucleic acid to amplify the viral RNA followed by subsequent separation and visualization.

ELISA is one of the sensitive tests for detection of virus. In the present study, DAC-ELISA was performed using different dilutions of primary antibody (monoclonal antibody from Agdida Ltd, USA) to determine antibody titre. Absorbance value read in ELISA reader were compared with that of healthy samples. The dilution of 1:200 gave the highest mean absorbance value (nearly three times more than that of healthy sample) compared to other dilutions *viz.*, 1:100, 1:300 and 1:500. In ELISA, generally the replicated samples having mean absorbance value which is double that of healthy sample was considered as infected. Accordingly, the BBrMV infection could be easily detected up to maximum antiserum dilutions of 1:500 and this was previously reported by Unnithan, 2008. Dhanya *et al.* 2007 also determined titre for polyclonal antibody was 1:502 with mean absorbance value which was double that of healthy sample. DAC-ELISA was again performed to detect BBrMV with the previously determined antiserum titre (1:200) which gave clear difference between healthy and infected sample with the absorbance value of infected samples almost thrice as that of healthy samples. Many workers successfully detected BBrMV in infected samples by ELISA (Thomas *et al.*, 1997; Krishna Reddy *et al.*, 2000).

DIBA was conducted to detect the presence of virus in infected samples by using monoclonal antibody specific to BBrMV. The infected leaf sample gave positive reaction for DIBA and this could be detected by the purple coloured spots on nitrocellulose membrane which was absent in healthy samples and the buffer check. DIBA was found to be more effective than Indirect-ELISA for the detection of potyviruses (Bhat *et al.*, 1999). It was very convenient because sample preparation and the subsequent procedures for virus detection were simple and sophisticated instruments such as an ELISA-reader or a Thermo cycler are not required. This could be easily applicable at field level also. A field level diagnostic kit based on DIBA was developed for detection of *Cassava mosaic geminivirus* (CMG) and it was able to obtain the result within 4 h (Nair, 2012).

DIBA and ELISA are solid phase serological detection methods and depend on the concentration of the virus particles. The sensitivity of DIBA in relation to

ELISA depends on the ELISA format, type of plant tissue sampled, plant sap components as well as the virus itself. Both ELISA and DIBA could be considered as efficient methods for detection of BBrMV, although probability of non-specific reaction of antibody and components of plant sap was higher in DIBA than in ELISA (Dhanya *et al.*, 2007).

5.3 NUCLEIC ACID BASED DETECTION OF BBrMV

Good quality RNA is a prerequisite for a reliable RT-PCR and PCR reaction. Young leaves were most suitable than older leaves for RNA isolation. This might be due to reduced amount of tannins and phenolics in the younger leaves compared to older leaves. However, there are many methods standardized for total RNA isolation. In the present study, two protocols using different reagents *viz.*, Ambion Purelink RNA and trizol reagent were used for isolation of RNA. The protocol using Ambion Purelink RNA reagent provided highest quality and quantity of RNA as revealed by spectrophotometer analysis and gel electrophoresis compared to trizol reagent protocol. The same type of tissue samples at different growth stages may require a special protocol for total RNA isolation which might due to the variability in compositions and content of the chemical (Wang *et al.*, 2007). Although RNA yield obtained from leaf samples using Ambion and trizol reagent was almost similar, but the Purelink RNA reagent provided higher quality RNA as indicated by $A_{260/280}$ ratio. The traditional trizol method which is the most common RNA isolation method was easy to conduct and saved time. However, the low $A_{260/280}$ ratio indicated that RNA isolated by this method might contain chemical substances, such as phenols, polysaccharides and salts, which could lead to negative effects (Zhang *et al.*, 2013). Among the two methods evaluated for RNA isolation from leaves, Ambion Purelink RNA reagent method provided highest quality and quantity of RNA. Hence this reagent allowed isolation of high quality RNA from plant tissues, especially those rich in polyphenolics or starch like in banana, potato tuber and other tuber crops. Manasa (2014) also reported that the Ambion Purelink RNA

reagent method provided good quality of RNA than trizol method in Greater yam (*Dioscorea alata*) which was also rich in polyphenols and starch.

RT-PCR was the most reliable and sensitive assay for the detection of virus in the infected banana plants even at low virus concentration. In the present study, leaf samples were also tested by RT-PCR to ensure that the plants with low virus load were not neglected. The primers Bract1 and Bract2 which were used to amplify the partial CP gene yielded an expected product of size ~604bp (containing C terminus of the coat protein-coding and 3' untranslated region) in all positive samples. Previously, RT-PCR analysis with these primers had been successfully used for the detection of BBrMV infecting banana from Coimbatore and Tiruchirappalli regions of southern India (Rodini *et al.*, 1997). Similarly, Rodini *et al.*(1999) detected BBrMV in five different countries in South East Asia using same primer yielding expected product of size ~604 bp (containing C terminus region of the coat protein-coding and 3' untranslated region).

The amplified genome products of BBrMV were cloned and sequenced. The BLAST analysis revealed that the present isolate showed highest homology of about 98 per cent to the Coimbatore isolate (KF385491). These primers could be utilized for the detection of BBrMV from the field samples for routine survey purpose. There were many reports of utilizing RT-PCR and variants of RT-PCR for the detection of BBrMV strains and cloning of CP of BBrMV genome segments. Bateson and Dale (1995) obtained 604bp of CP of BBrMV and while Thomas *et al.*, (1997) obtained only 340bp region of coat protein.

5.4 AMPLIFICATION AND CLONING OF COAT PTOTEIN (CP) GENE OFBBrMV

The CP coding region of members of *Potyviridae* had been mainly used to establish evolutionary relationships at both species and strain levels primarily because the majority of potyvirus sequences on the databases were derived from this region and it was multifunctional, played important roles in virus life cycle and virus-vector interaction (Adams *et al.*, 2005). Among the viruses belonging to

the family Potyviridae, species identification was based on genetic information, host range, method of transmission, and antigenicity (Berger *et al.*, 2005). The genetic information mainly based on CP region such as: (A) the amino acid sequence identity of the CP should be less than 80 per cent. (B) nucleotide sequence identity should be less than 85 per cent over the whole genome and (G) the polyprotein cleavage site should be different from that of other species; therefore, it could be a target of selection in the present study.

Molecular cloning of many plant viruses had been documented. One main objective of cloning plant viruses has been the improvement of virus detection and diagnosis (Jelkmann *et al.*, 1989). RNA cloning is usually done by reverse transcription into cDNA followed by insertion into plasmid or lambda vectors and transformation of strains of *E. coli* (Maniatis *et al.*, 1982).

Rodini *et al.*, (1999) amplified partial coat protein gene with 604 bp product including the CP-coding region and 3' untranslated region (UTR) region of genome by using specific primers. The amplified product was cloned and sequenced. In the present study, a pair of species specific primers (BCF1/R1) was designed to detect BBrMV and used in the PCR based strategy to characterize the entire CP region of the virus. Forward and reverse primers were designed to amplify the coat protein gene of BBrMV based on multiple sequence alignment (MSA) of CP gene sequences available from the NCBI GenBank database. MSA is a tool to determine levels of homology, and hence relatedness between members of a series of globally related sequences would be inferred. When designing primers, the conservation of the region, the degeneracy of the genetic code and parameters of PCR reaction must be considered. In MSA, sequences were aligned optimally by bringing together the greatest number of similar characters into register in the same column of the alignment. Multiple sequence alignment could do both in the nucleotide and amino acid levels. When conserved sequence as not present in the nucleotide level or when the DNA sequence was not available, multiple sequence alignment in the amino levels was taken into

consideration, accounting the degeneracy of genetic code. In this study, conserved sequences could be detected at the nucleotide level itself for primer designing. The forward primer (5′GATGATGACCCAAGCCGC3′) and reverse primer (5'GCAGAGAG GCATATCAC 3') were derived from conserved region of CP gene based on optimal primer character. The key to the PCR lies in the design of primers set. It is essential to consider several parameters *viz.,* the length of the primer, self-complementarity, expected PCR product, GC content, melting temperature, feasible annealing temperature and probability for primer dimer formation, hairpin formation and other parameters are need to be optimized for successful PCR. These parameters could be easily optimized by *in silico* methods with widely available computer programs before synthesis of the primer (Elsalam, 2003). In present investigation, OligoAnalyzer 3.1(Integrated DNA technologies) was used to validate selected primer before synthesis. The analysis of primers done by using OligoAnalyzer 3.1 program revealed that it possessed the optimal primer parameters such as GC content and annealing temperature and also the designed primer did not showed any hairpin formation and self dimer. Similarly, Siljo *et al.*(2012) designed Potyvirus specific primer to amplify coat protein of BBrMV, forward and reverse primers were designed corresponding to the conserved region and identified based on multiple sequence alignment of CP gene and 3′ untranslated region (UTR) of all available BBrMV sequences from the GenBank database.

RT-PCR analysis with these primers yielded an expected product of size ~850 bp in the positive samples. These products were cloned and sequenced and the CP sequence obtained with these primers was used for *in silico* analysis. Sankaralinga *et al.* (2006) amplified CP gene of Indian BBrMV isolate which consisted of 1062 nt including 900 nt of the CP coding region and 162 nt from the 3' UTR. Balasubramanian and Selvarajan (2012) also amplified, cloned and sequenced the complete genome of an Indian isolate (TRY) of BBrMV from the French plantain cv. Nendran (AAB).

The CP sequence obtained with these primers was used for phylogenetic analysis which could be used study genetic diversity. As CP gene sequences are used to develop the pathogen derived resistance against *potyvirus* by means of genetic engineering, BBrMV diversity could help in predicting the risk of resistance breakdown in the developed resistant transgenic banana lines. Hence the efficient long term management strategies could be achieved by preventing the loss-of resistance of CP mediated virus resistant due to the evolution of new variants. CP gene sequence generated in the present study was compared with 22 previously reported isolates originating from different geographical region. Phylogenetic tree was constructed from CP gene sequences showed two monophyletic clusters in the world population of BBrMV. However, the Indian isolates did not show any relationship according to geographical origins and the hosts from which they were isolated. This finding is in consistence with the results of Balasubramanian and Selvarajan (2014) who reported, using phylogenetic analysis based on CP coding region of 49 BBrMV isolate, revealed that there is much diversity among Indian isolate and did not show any relationship according to geographical origins. A probable reason for the geographical distribution is that the virus has moved as a separate event. Perhaps through different infected cultivars of banana (Rodoni *et al.* 1999). BBrMV was first noticed in 1966 (Samraj *et al.*, 1996) and because of prolonged presence, high divergence of BBrMV populations might have occurred. Though, BBrMV was noticed first in southern parts of Kerala, it had moved to other neighboring states *viz.*, Andra Pradesh, Tamil Nadu and Karnataka during the past five decades either through infected planting material or through aphid vector. There is no domestic quarantine enforced to restrict the movement of banana suckers between the states. This virus had recently been reported to infect small cardamom which was grown along with banana in Western ghat region of Kerala and Karnataka (Siljo *et al.*, 2012).

As banana is a vegetatively propagated crop, there is an increased chance of virus introduction into new areas through the planting material. Naturally infected

banana plants might also become the source of inoculum when used as planting materials. The present study validated the detection techniques of *Banana bract mosaic virus* infection in Banana and could be used for perfecting the technique of virus indexing. Molecular clones of CP gene were developed which helped in characterization and also would help in production of good quality antiserum through recombinant DNA technology.

Future line of work

Molecular clones of CP gene could be used for the production of antiserum through recombinant DNA technology and these could be used as disease diagnostic probes for more sensitive molecular techniques like Nucleic acid spot hybridization. Information by characterization of CP gene could be used for development of virus resistant transgenic banana lines through coat protein mediated resistance.

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SUMMARY

6. SUMMARY

The study entitled "Molecular cloning and characterization of coat protein gene of *Banana bract mosaic virus*" was conducted at Center for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during 2015-2016. The present study was undertaken to clone and characterize the coat protein gene of virus at molecular level. Banana (*Musa* spp.), identified as a 'tropical treasure' is the most remunerative fruit crop of Kerala and plays a pivotal role in the income security of farmers. But, the diseases especially viral, infecting the crop is the major hurdle for its profitable cultivation. The disease caused by BBrMV is having devastating effect on the production of banana in major banana growing countries including India.

In this study, the infected suckers were collected from Banana Research Station (BRS), Kannara and maintained in Virology net house, Department of Plant Pathology as virus culture for further characterization of virus.

The various symptoms of disease were recorded under field condition during collection of samples. The symptoms observed were longitudinal, irregular, reddish streaks of varying sizes on the psuedostem, mosaic pattern on the bracts, fan like arrangement of leaves (resembled like a travellers palm), spindle shaped lesions and streaks on leaves and malformed and under sized fruit.

Serological (DAC-ELISA and DIBA) and nucleic acid based (RT-PCR) techniques were carried out to detect the presence of BBrMV infection. DAC-ELISA was done by determining the antibody titre with different dilution of (1:100; 1:200; 1:300; 1:500) and it was found that BBrMV could be the best detected in 1:200 primary antibody dilution along with 1:500 alkaly-phosphate conjugate secondary dilution. The DAC-ELISA was performed by a titre of antiserum at 1:200 which was previously determined by DAC-ELISA and it revealed that the BBrMV specific antibody gave higher reactivity and it can gave clear difference between healthy and infected sample.

DIBA was performed to detect BBrMV, which gave positive reaction for infected leaf sample and this could be detected by the purple coloured spots on nitrocellulose membrane unlike those produced by the healthy ones. Among the solid phase serological detection methods such as DIBA and ELISA, DIBA was simple and convenient for field application since no sophisticated instruments such as ELISA-reader are required.

Two different protocols using Ambion Purelink RNA reagent and TRIzol reagent were used for isolation of RNA. The Ambion Purelink RNA reagent method was found to be most appropriate for RNA isolation from banana leaf since provided highest quality and quantity of the RNA. For nucleic acid based detection, RT-PCR was performed by using CP gene specific primers of B1 and B2 were used which produced amplicons of ~605 bp in infected samples.

The species specific primer pair was designed to amplify of the coat protein gene of virus based on the most favorable combination of conserved regions in the multiple aligned nucleotide sequences and named as BCPF1 and BCPR1 respectively. The primer set was validated for optimal primer characters by using OligoAnalyzer 3.1 program and standardized annealing temperature of 58^0 C using gradient PCR. RT-PCR analysis with this primer yielded an amplicon of ~850 bp in infected samples. The positive samples were gel eluted and cloned into E. coli DH5α. The presence of gene insert in transformed colonies was confirmed by colony PCR using plasmid specific primer (T7 and SP6) which yielded amplicons of expected band size of 1150 bp. This amplified product was sent for sequencing to obtain CP gene of BBrMV.

The blast analysis of CP coding region of the virus has maximum homology of 99 percent to KER2 isolate (Kasargod, Kerala; accession KF385491). The sequence exhibited significant nucleotide identity (99 to 96 per cent) and amino acid identity (95 to 83 per cent) with other BBrMV nucleotide and protein sequence of BBrMV in the database. The Phylogenetic analysis by the alignment of CP gene sequences of 23 isolates also revealed that the present isolate was

more similar to KER2 isolate which is clustering with TN13 (KF385477; Tanjore, Tamil Nadu).

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7. REFERENCES

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ANNEXURE

ANNEXURE I

List of laboratory equipment used for the studies

Electrophoresis unit   :   Biored

Thermocycler   :   Agilent

Laminar air flow   :   Labline industries

Shaker incubator   :   JEIO Tech, Korea

ELISA reader   :   VERSA max, USA

High speed refrigerated centrifuge   :   Kubota

Geldoc XR   :   Biored

ANNEXURE II

Chemical composition of buffers used for ELISA and DIBA

1. **Coating buffer (PBS - pH 7.4)**

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

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

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

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

Diethanolamine (9.8 %)	98 ml
Sodium azide	0.2 g
Water 800 ml Add HCI to give pH 9.8	

4. **Blocking solution**

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

5. **Antibody dilution buffer**

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

ANNEXURE III

Chemical composition of buffers and dyes used for gel electrophoresis

1) Loading/tracking dye

Bromophenol blue	0.25%
Xylene cyanol	0.25%
Glycerol	30%

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

2) Ethidium bromide (intercalating dye)

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

3) TAE buffer (pH-8.0) – 50X

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

The solution was prepared and stored at room temperature

4) MOPS buffer (pH-7) – 10X

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

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

MOLECULAR CLONING AND CHARACTERISATION OF COAT PROTEIN GENE OF *BANANA BRACT MOSAIC VIRUS*

by

DARSHAN GOWDA M. R.
(2014-11-233)

ABSTRACT

Submitted in partial fulfilment of the requirement

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ABSTRACT

Abstract

Banana (*Musa* spp.), identified as 'tropical treasure' is grown extensively in the tropical and sub tropical regions of the world. Diseases, especially those caused by viruses are major constraints for the profitable cultivation of banana. Among the viral diseases, banana bract mosaic is one of the most important, which leads to an yield reduction ranging from 52 to 75 per cent. This disease is caused by *Banana bract mosaic virus* (BBrMV) which is a member of genus *Potyvirus* and family *Potyviridae*. In case of any viral disease, early diagnosis is very important since symptomless hosts carry the viral inoculum. Development of molecular clones of viral genome has immense application in the field of disease diagnostics and management. Hence, the present study was carried out with the objective to develop molecular clones of coat protein (CP) gene of BBrMV and to characterize it.

The infected suckers were collected from Banana Research Station (BRS), Kannara and maintained in the insect proof net house of Department of Plant Pathology. The symptoms developed on different plant parts under natural field conditions were documented which included longitudinal, irregular, reddish streaks of varying sizes on the base of pseudostem, mosaic pattern on bracts, fan like orientation of leaves, spindle shaped lesions on leaves, reduced bunch size and malformed fingers.

The serodiagnostic technique namely, Direct Antigen Coating-Enzyme linked immuno sorbent assay (DAC-ELISA) was validated by determining the antibody titre with different dilutions of primary antibody *viz.,* 1:100, 1:200, 1:300, 1:500 and it was found that BBrMV could be best detected at 1:200 dilution along with 1:500 dilution of secondary antibody. Later, the presence of virus particles in the samples were confirmed by DAC-ELISA using the standardized combination of primary and secondary antibody dilutions. Dot Immuno Binding Assay (DIBA) was validated to detect BBrMV and showed positive reaction for infected leaf sample which was detected by the development of purple coloured spots on nitrocellulose membrane.

The genome of BBrMV is RNA and hence, molecular detection of virus was standardized by Reverse Transcription- PCR (RT-PCR). Total RNA was isolated by two different protocols using different reagents. Among the two methods, the one with Ambion Purelink RNA Reagent was the most appropriate for RNA isolation from banana since it provided highest quality and quantity of RNA compared to the protocol with TRIzol reagent. The isolated RNA was converted into complementary DNA (cDNA) using First Strand cDNA synthesis kit. RT-PCR amplification of coat protein gene was standardized using gene specific reported primer (B1/B2) and designed primer (BCPF1/R1) which yielded amplicons of approximate size, 605 bp and 850 bp respectively.

The molecular cloning of CP gene was done in *Escherichia coli* (DH5-alpha). The presence of gene insert in transformed colonies were confirmed by colony PCR using plasmid specific primer (T7 and SP6) which yielded amplicons of expected band size of 1150 bp. The amplified colony PCR products were sequenced to obtain CP gene sequence of BBrMV.

The characterization of cloned CP gene of BBrMV was carried out by *in silico* analysis. The blast analysis revealed that the CP gene sequence of the virus showed maximum homology of 99 per cent to KER2 isolate from Kasargod, Kerala (Accession no. KF385491). The sequence exhibited significant nucleotide identity (99 to 96 per cent) and amino acid identity (95 to 83 per cent) with other nucleotide and protein sequences of BBrMV available in the database of Genbank. The phylogenetic analysis by the alignment of CP gene sequences of selected 22 isolates also revealed that the present isolate was more similar to KER2 isolate and the Indian isolates did not show any relationship based on geographical origin.

The recombinant clones developed in the present study could be applied in serodiagnosis and genetic engineering. This could be also used as disease diagnostic probes for more sensitive molecular diagnostic techniques like Nucleic acid spot hybridization.