IMMUNOLOGICAL AND MOLECULAR DETECTION OF BANANA VIRUSES AND PRODUCTION OF DISEASE FREE PLANTING MATERIALS

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(2011-11-110)

Thesis submitted in the partial fulfillment of the requirement

for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture

Kerala Agricultural University, Thrissur

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VELLAYANI, THIRUVANANTHAPURAM - 695 522

KERALA, INDIA

2014

DECLARATION

I hereby declare that this thesis entitled 'immunological and molecular detection of banana viruses and production of disease free planting materials' is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associate ship, fellowship or other similar title of any university or society.

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ACKNOWLEDGEMENT

This thesis will be incomplete without expressing my gratitude to:

God almighty for the blessings showered upon me in all situations.

I express my deep sense of gratitude and indebtedness to my major advisor and chairman of the advisory committee, Dr. K. Umamaheswaran for his valuable guidance, sincere help, support, patience and encouragement throughout the course of this study.

I sincerely extend my profound gratitude to Dr. K. K. Sulochana, Professor and Head, Dept. of Plant Pathology, College of Agriculture, Vellayani for her support, valuable suggestions and constant encouragement during the course of investigation.

I extend my gratitude to Dr. C. Gokulapalan, Professor, Dept. of Plant Pathology, College of Agriculture, Vellayani for the support, timely help and co operation during the course of the study.

I would like to express my sincere gratitude to Dr. K. B. Soni., Associate Professor, Dept. of Biotechnology, COA, Vellayani for her valuable suggestions, wholehearted help and guidance throughout the conduct of my study.

My sincere thanks to Dr. C A. Mary, Dr. Naseema, Dr. Lulu Das, Dr Geetha, and Dr. V. K. Girija for their whole hearted help throughout the research work.

I thankfully remember the professors of College of Agriculture, Vellayani, Dr. Anith, K. N., Dr. Meena Kumari., Dr. Sajitha Rani., Dr. Chandini, S., Dr. Hebsey Bhai, Dr. Arther Jacob and Dr. Shalini Pillai for their help and support during the course work and research work.

I thank my seniors Lekshmi chechi, Asha Chechi, Aysha chichi and Reshmi chechi for their affection and encouragement during my study at COA, Vellayani.

Special thanks to my friends, Revu, Manal, Ashish, Anand, Manasa for the most sincere help rendered in different stages of the programme.

I gratefully acknowledge Sri. C.E. Ajith Kumar, Dept. of Agri. Statistics for the statistical analysis of my research data.

I am thankful to Dr. Sverup John, Dean, COA, Vellayani for the infrastructural facilities, resources and academic atmosphere extended to me during the course of my study.

I am grateful to Kerala Agricultural University for the senior fellowship and contingency grant allotted to me during the course of the study.

Finally, I would like to express my sincere gratitude to my parents, my husband, my brother, my son Aabid and other family members for their selfless support, unbound love and encouragement all through my life.

Aliya Ferzana

Dedicated to My parents &

My husband

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LIST OF ABBREVIATIONS AND SYMBOLS USED

%	Per cent
µm	Micro meter
µl	Micro litre
@	At the rate of
°C	Degree Celsius
CD	Critical difference
cm	Centimeter
nm	Nano meter
APS	Ammonium per sulphate
DAC - ELISA	Direct antigen coating – ELISA
et al.	And other co workers
Fig.	Figure
DAS - ELISA	Double antibody sandwich - ELISA
N	Normal
g-1	Per gram
i.e.	that is
TEMED	Tetra methyl ethylene diamine
TBS	Tris buffer saline
l.	Litre
m	Meter
mm	Milli meter
mg	Milli gram
ml	Milli litre
rpm	Rotations per minute
sec	Seconds
BBTV	*Banana bunchy top virus*
BBrMV	*Banana bract mosaic virus*

BSV	Banana streak virus
CMV	Cucumber mosaic virus
viz.	Namely
sp. or spp	Species (Singular and plural)
var	Variety
PCR	Polymerase Chain Reaction
cv.	Cultivar
RT-PCR	Reverse transcription – PCR

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Introduction

1. INTRODUCTION

Banana is one of the most important fruits in the world, and it is gown in India with great socio economic significance. India is the largest producer of banana in the world with a total production of 191million tonnes from an area of 0.64 million ha (Selvarajan *et al.,* 2011). In Kerala, the area under banana cultivation is 59,069 ha and the production is 5,14,054 tonnes.

Banana is a perennial herb, 2-9 m in height, evolved in South East Asia, New Guinea and Indian sub continent. The propagating materials of banana are suckers, sword suckers are preferred to water suckers for planting in new fields because of their superior quality and yield.

The major production problems in the banana cultivation are the prevalence of viral diseases and other pathological and entamological problems, making difficulty in the availability of pest and disease free suckers. Among these, viral diseases are highly problematic. Virus infecting banana can have a direct effect on production by reducing plant growth and yield. They can also have important indirect effect by restricting germplasm movement and plants to damage by other biotic and abiotic stress factors. Four important viral diseases are reported in banana, viz. banana bunchy top disease (BBTD), banana bract mosaic disease (BBrMD), banana streak disease (BSD), and Cucumber mosaic disease (CMD). All these diseases are transmitted primarily through infected suckers and secondarily through vectors.

BBTD caused by *Banana bunchy top virus* (BBTV) is a very serious disease in India. Since the 1970, BBTD has devastated large plantation of cv *Virupakshi* (AAB), in lower Pulney hills, Tamil Nadu (Kesavamoorthy, 1980). The area under cultivation was reduced from18000 ha to 2000 ha. BBTV was first reported in Fiji in 1889. BBTV is a multicomponent circular ssDNA included in the genus *Babuvirus,* family: *Nanoviridae.* It is transmitted by banana black aphid, *Pentalonia nigronervosa.* BBTV infection can result in 100% yield loss.

BBrMD, locally known as 'kokkan' in Kerala was first observed in Nendran cv. by Samraj *et al.* (1966) in Thrissur district of Kerala. BBrMD caused

by *Banana bract mosaic virus* (BBrMV), a non persisantly aphid transmitted virus of banana, cause yield reduction up to 40% (Rodoni *et al.,* 1997). BBrMV has been classified as a member of genus '*Potyvirus'* in the family: *Potyviridae.* The potyvirus genome consists of a positive sense single strant RNA (ssRNA) molecule of approximately 10Kb size.

BSD, now occurring in almost all banana growing areas has become major menance to the commercial banana group 'poovan' (AAB). BSD caused by *Banana streak virus* (BSV) is a para retro virus included in the genus of *Badnavirus* belonging to the family *Caulimoviridae* (Selvarajan *et al.,* 2011). BSV is transmitted by citrus mealy bug (*Planococcus citri*) and also through infected suckers. BSV has a circular double stranted DNA (dsDNA) genome. Estimated yield losses of between 7 to 90% have been attributed to this disease (Harper *et al.,* 2004). BSV was reported in 1996 to occur in India (Selvarajan *et al.,* 2011). BSV exist in two forms. episomal form and an integrated form. The integrated viral sequence of BSV genome may become the source of BSV infection brought by stress factors such as environmental condition and *invitro* micropropagation.

Cucumber mosaic virus (CMV) causing cucumber mosaic disease is an important virus belonging to the genus *Cucumovirus* and family *Bromoviridae.* CMV causes great losses in vegetables, ornamentals, and fruits and it is destructive due to rapid spread by more than 60 aphid species in field. CMV is transmitted by aphid species (*Aphis gossypi and Myzus persica*) in a non persistant manner, which colonize wide range of plants but not banana. CMV is a multi component single stranded RNA (ssRNA) virus and has the broadest host range, infecting more than 1000 species of plants including banana. CMV was first reported in Australia by Magee in 1930 (Magee, 1940). In India CMV was first reported at Maharashtra in 1940 (Selvarajan *et al.,* 2011). CMV infecting banana resulted in reduction of bunch weight between 45% and 62% (Estelitta *et al.,* 1996).

The above described four viruses cause considerable economic losses to banana cultivation. Control strategies targeted towards viruses are more difficult

to design and implement, as no anti viral treatments are readily available and source of natural resistances are scarce. Therefore the use of healthy planting materials is currently the most effective and cheapest way to avoid the spread of viral disease. In this regard, *in vitro* micropropagation is particularly suited for the development of such control strategies. In addition, use of tissue culture to propagate banana has been reported to trigger episomal BSV expression in Musa hybrids with B genome.

Taking into consideration of above aspects and also the importance of BBTV, BBMV, BSV and CMV in banana cultivation, this study was undertaken to investigate symptomatology, host pathogen interaction, serological and molecular diagnosis of viruses and production of virus indexed tissue culture plantlets.

Review of Literature

2. REVIEW OF LITERATURE

India is the home for bananas and is being grown even before the Vedic times. Banana is referred as "Kalpatharu" (Plant of Virtue) due to its multifaceted uses. In terms of nutritional value, the banana is rich in carbohydrates, minerals and vitamins. It is widely cultivated in varying agro climatic regions. However, banana cultivation continues to face several pests and diseases which affect the production and productivity (NRCB, 2011). Four viruses are known to naturally infect banana viz. BBTV, BBrMV, BSV and CMV (Rajasulochana *et al.*, 2008)

Banana bunchy top disease caused by BBTV is the most economically important disease of banana in Asia, Africa and South Pacific (Dale, 1987). *Banana streak virus* (BSV), the causal agent of banana streak disease of *Musa* spp. is of worldwide occurrence and is now considered to be the most frequently occurring virus (Lockhart, 1997). Estelitta *et al.* (1996) reported that *Cucumber mosaic virus* infecting banana is an emerging threat to the cultivation of banana in Kerala, India, especially where cucurbitaceous vegetables are cultivated as intercrops in banana.

2.1 SYMPTOMATOLOGY

Nelson (2004) described the symptom of BBTD in suckers, maturing plant, morse code streaking symptom and green 'J' hooks symptom. The suckers which developed from infected mother plant were usually severely stunted; leaves did not expand normally and remained bunched at the top of the pseudostem. The leaves were stiff, erect, shorter, and narrower than normal leaves. Suckers with this symptom did not bear fruits. In infected maturing plant, leaves were narrow, wavy and had chlorotic leaf margin. Severely infected plants did not bear fruits. Some infected plants produced fruits with distorted hands and fingers. The initial symptoms due to BBTV infection are Morse code streaking and green 'J' hook symptom. Morse code symptom described as dark green in the veins of lower portion of the leaf midrib and the leaf petiole. Green 'J' hook symptom described

as hook like extension of the leaf lamina veins.

Magee (1927) reported that the first symptom of the disease was the appearance of dark green streaks in the under surface of leaf. Thomas *et al.* (1995) described BBTV symptom in *invitro* banana plantlets as green flecks in the leaf sheath, chlorotic to translucent flecks on the leaf, dark streaks in the leaf. Also in vitro plantlets with BBTV infection were chlorotic and less vigorous than healthy plantlets. The concentration of BBTV in older leaves with moderate symptoms was lower than that in younger leaves with obvious symptoms (Zhang *et al.*, 1995).

The symptom of BBrMV in Musa balbisiana, Klue tetrapod from Coimbatore region include distinctive streaks in the bract of banana inflorescence and mosaic on petiole of leaf (Rodoni *et al.*, 1997). Thomas and Magnaye (1996) described symptom of BBrMV as characteristic dark reddish-brown mosaic pattern on the bracts of the inflorescence. Initial symptoms include green or reddish-brown (depending on cultivar) streaks or spindle shaped lesions on the petiole. The petiole showed tendency towards a congested leaf arrangement. Leaf lamina symptoms were more prominent on the younger leaves in recent infections. Leaf lamina symptoms consist of spindle-shaped chlorotic streaks running parallel to the veins. After the removal of dead leaf distinctive dark coloured mosaic patterns, stripes or spindle-shaped streaks were visible in the pseudostem. Chlorotic streaks were also present on the bunch stalk. High disease incidence was associated with increased levels of fruit rejection on commercial plantations. Symptoms of BBrMV includes distinct discontinuous streaks along the primary veins, which appear to be irregularly thickened, and scattered white to yellowish streaks running from the midrib to the leaf margin. After removal of the dead leaf sheath, spindle-shaped streaks or stripes, sometimes with mosaic patterns were visible on the pseudostem (Magnaye and Espino, 1990).

Lockhart (1986) described symptoms of BSV as continues or broken chlorotic leaf streak and spindle shaped lesion that may be sparse or concentrated in distribution and become progressively necrotic. Foliar symptoms caused by BSV initially resemble those caused by CMV. Typical symptom of BSV includes

yellow streaks on leaves, splitting of the pseudostem and abnormal emergence of the bunch from the middle of the pseudostem, bearing fewer fruits of smaller size (Jones and Lockart, 1993). However necrotic streaks later develop in the leaves of plants infected by BSV, which are not usually seen in the leaves infected by CMV. Cherian *et al.* (2003) observed chlorotic streak and black streak as symptom of BSV in two accession BRS1 (AAB) and Mysore poovan (AAB). The symptom expression of BSV depends on environmental factors (Leureux, 2003). Dahal *et al.* (2000) described the symptoms of BSV infecting different *Musa* genotypes in Nigeria. Symptoms included discrete yellow streaks on the leaves, internode shortening with rosette-like leaves, a necrotic cigar leaf and distorted (inverted) bunch. They also observed that symptom expression was more severe during rainy season. Wambulwa *et al.* (2013) reported that Mysore virus isolate of *Banana streak virus* (BSMysV) and the banana cultivars containing the balbisiana (B) component were associated with the most severe banana streak disease symptoms. Ndowora *et al.* (2000) described symptoms of BSD as yellow and necrotic leaf streaks, lethal stem necrosis, pseudostem splitting and 'choking'. Karanja *et al.* (2013) studied the effect of environment in the symptom expression of BSD in Kenya. They found that BSD symptom expression was significantly influenced by plant age, cultivar, season and growth site. The symptom expression of BSV varied with temperature (Dahal *et al.*, 1998).

Most strains of the CMV did not produced severe symptom or caused significant crop damage. The symptom of CMV consists of foliar mosaic, occasional leaf deformation, especially in young suckers developing from infected mother plant. Mosaic symptoms were more pronounced during cool weather but do not persist. The heart rot strains of CMV caused damaging symptoms, which include chlorosis, cigar leaf necrosis, internal pseudostem necrosis and plant death (Lockhart, 2002).

2.2 HOST - PATHOGEN INTERACTION

2.2.1.1 CARBOHYDRATES

Nair and Wilson (1970) reported higher percentage of carbohydrates in the leaves of banana bunchy top infected plants than the healthy plants. Alagiamanavalan *et al.* (1973) reported that there was an increase in reducing and non – reducing sugars and pectin content in BBTV affected fruits. Rajagopalan (1980) found that carbohydrate content of bunchy top infected banana leaves showed an increasing trend in early stages of infection compared to healthy. But in later stages, it was vice versa.

Total carbohydrate content of BBrMV infected leaves were less than healthy ones at three month after planting (MAP), six MAP. and flag leaf stage (Dhanya,.2004). Smitha (2001) reported higher carbohydrate content in the leaves of healthy banana compared to that of BBrMV infected plants. But in bract and flag leaf, carbohydrate content was more in diseased plants.

Shalitin *et al.,* (2000) reported that the levels of primary sugars (Glucose and Fructose) were significantly higher in CMV infected *Cucumis melo* plants than in healthy plants.

2.2.1.2 CHLOROPHYLL

The most obvious symptom of systemic virus infection is the mosaic pattern on the leaves. Yellowing and chlorosis is the characteristic sign of the altered photosynthetic activity. Virus infection effects photosynthesis in a complex manner, depending on the particular host-virus combination (Almasi *et al.*, 2001).

Hook *et al.* (2007) reported that chlorophyll a and chlorophyll b content in banana leaves infected with BBTV was significantly reduced compared to healthy plant. Yasmin *et al.* (2001) investigated the effect of BBTV on leaf chlorophyll content of banana and found that due to the infection, there was

severe reduction in total chlorophyll, chlorophyll a, chlorophyll b over healthy control.

Total chlorophyll content was found to be less at all stages of BBrMV infected banana leaves than healthy ones (Dhanya, 2004).

Devi and Radha (2012) observed reduction in chlorophyll a, chlorophyll b and total chlorophyll in cucurbits plants infected with CMV. El-Deeb *et al.* (1997) studied the effect of CMV in the ultrastructure of banana mesophyll cells. They found that chloroplasts of CMV-infected cells showed swollen granal thylakoids and in some cases, they appeared swollen and degenerated. In other cases, chloroplasts contained vesicles and osmophilic granules and an abundant number of mitochondria appeared in CMV-infected cells.

Total chlorophyll, chlorophyll a and chlorophyll b were found to be lower in bottle gourd plants infected by three viruses namely *Bottlegourd mosaic virus* (BgMV), *Watermelon mosaic virus* 2 (WMV2) and *Papaya ring spot virus* (PRSV), and also observed that ratio of chlorophyll a to b was higher in all three virus infected samples (Muqit, 2007). Pazarlar *et al.* (2013) reported that the total chlorophyll was reduced due to *Tobacco mosaic virus* (TMV) in *Capsicum annuum.* Palaniswamy *et al.* (2011) reported that *Yellow vein mosaic virus* infection in okra resulted in reduction of chlorophyll a, chlorophyll b and total chlorophyll content than healthy ones.

2.2.1.3 PHENOL

Total phenol was reported to be high in virus infected leaves of many plants (Srivastava and Tiwari, 1998). Rajagopalan (1980) reported reduced accumulation of total phenol in BBTV infected banana plants.

Smitha (2001) reported that the BBrMV infected plants had more phenol content than healthy ones. Dhanya (2004) observed increased phenol content in BBrMV infected banana leaves than healthy ones in all growth stages.

The total phenols in cucumber plant were significantly increased as a consequence of *Cucumber mosaic virus* (CMV) infection (Devi and Radha, 2012).

Kato *et al.* (1993) extracted and characterized two phenolic compounds from cowpea leaves infected with cucumber mosaic virus. Saveetha *et al.* (2010) while investigating physiological changes due to virus infection reported increased phenol contents in finger millet plants infected by *Mottle streak virus.*

2.2.1.4 PROTEIN

Estelitta (1998) reported higher percentage of protein in banana bunchy top affected plants than healthy plants.

BBrMV infected leaves were found to have more protein content than healthy ones in all growth stages. Both healthy and infected samples showed decreasing trend in protein content in all growth stages till bunch formation (Dhanya, 2004)

CMV and pepper yellow mottle virus (PYMoV) infected leaves of pepper showed increased protein content than healthy ones in all growth stages. The highest protein content was recorded at 6 month after grafting. (Ayisha, 2010).

Protein content was found to be higher in cucumber plants infected with CMV. The protein content of healthy plant was 34 µg g-1 while in treated plants it varied from 36 to 59 µg g-1 (Devi and Radha, 2012).

Mohammed (2011) reported that *Beet mosaic virus* (BtMV) infected beet plants showed high significant increase in total protein content when compared with healthy beet plants.

2.2.2 DEFENCE RELATED ENZYMES

Dhanya (2004) observed increased peroxidase (PO), poly phenol oxidase (PPO) activities in BBrMV infected banana samples in all growth stages. Phenyl alanine ammonia lyase (PAL) activity was higher in infected samples in all stages except six month after planting. Smitha (2001) found enhanced activity of peroxidase, polyphenol oxidase and phenylalanine ammonia lyase in BBrMV infected banana plants compared to healthy ones.

Gomathi *et al.* (1993) observed enhanced activity of peroxidase, polyphenol oxidase and phenylalanine ammonia lyase in BSV and CMV infected banana plants.

Ayisha (2010) studied changes of peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) activities in CMV and PYMoV infected pepper samples. PO activity was increased in all stages except two month after grafting. Infected samples showed significantly higher PPO activity at all stages of analysis. Increased PAL activity was noticed during 4^{th} month after grafting and 6^{th} month after grafting.

2.2.3.1 SDS-PAGE (SODIUM DO-DECYL POLYACRYLAMIDE GEL ELECTROPHORESIS

Harding *et al.* (1991) analysed the molecular weight of coat protein of BBTV by doing SDS PAGE and silverstaining. The results revealed a protein with a molecular mass of 20 kDa. Purified BBTV preparations were analysed in 12 per cent sodium dodecyl sulphate polyacrylamide gel electrophoresis and visualized by staining with silver nitrate and found one major polypeptide of molecular weight 20.5 kDa, which migrated slightly slower than soybean trypsin inhibitor (Thomas *et al.*, 1991). Abdelkader *et al.*, (2004) performed SDS PAGE for determining size of purified coat protein of BBTV particles and found that the size is 21kDa.

The coat protein of the BBrMV resolved as one major polypeptide with an estimated molecular weight (M_r) of 38 kDa and three minor polypeptides with molecular weight of 63, 53 and 22 kDa, in a 12% SDS-PAGE (Kiranmai *et al.*, 2005).

Khan *et al.* (2011) performed SDS-PAGE to determine the size of coat protein (CP) in *Cucumber mosaic virus* (CMV) and the result revealed that the size was 25 kDa. Reichel *et al.* (1996) determined the molecular weight of the coat protein subunit of CMV using SDS-PAGE and it was found that the size was approximately 28 kDa.

2.2.3.2 ISOZYME ANALYSIS

Isozyme analysis is a powerful tool for estimating genetic variability identifying cultivars and germplasm accessions (Asiedu, 1992)

Dhanya *et al.* (2006) conducted native polyacrilamide gel electrophoretic analysis of isozyme in BBrMV infected plants as well as healthy plants and found single definite isoforms of polyphenol oxidase (Rm 0.63) in diseased plant.

2.3 IMMUNOLOGICAL AND MOLECULAR DIAGNOSIS

2.3.1 IMMUNOLOGICAL DIAGNOSIS

2.3.1.1 ELISA (ENZYME LINKED IMMUNOSORBENT ASSAY)

The enzyme-linked immunosorbent assay (ELISA) has been very popular for detection of viruses in plant material, insect vectors, seeds, and vegetative propagules since it was introduced to plant virology by Clark and Adams (1977). Prakash *et al.* (2010) reported that ELISA was good enough to discriminate BBTV, BBrMV, BSV, CMV infected suckers and micro propagated samples from healthy ones. El- Dougdoug *et al.* (2009) diagnosed BBTV and CMV by double antibody sandwich ELISA (DAS ELISA) using polyclonal antiserum. Thomas *et al.* (1995) reported that ELISA can be used for detection of banana plantlets in *invitro* plantlets. Zhang *et al.* (1995) detected BBTV by indirect ELISA using monoclonal antibody. Mariappan and Mathikumar (1992) reported that ELISA was the most sensitive method for detecting BBTV at very low virus concentration.

DAC-ELISA was found to be more economical serological method in detecting BBrMV (Dhanya *et al.*, 2007). A virus causing conspicuous dark streaks on the inflorescence bracts, purple coloured streaks on petioles and pseudostem, and occasional interveinal chlorotic streaks on leaves of banana in West Godavari (Andhra Pradesh, India) was purified and subjected to direct antigen coated

(DAC)-ELISA. The virus reacted positively with the antisera of *Bean yellow mosaic virus, Black eye cowpea mosaic virus, Lettuce mosaic virus, Pepper mild mottle virus, Pepper mottle virus, Peanut stripe virus, Sugarcane mosaic virus, Pea seed borne mosaic virus, Potato virus Y and Datura leaf distortion (DLDV) viruses.* Based on the result, the virus was identified as a *Potyvirus*, which might be similar to *Banana bract mosaic virus* reported from the Philippines (Kiranmai *et al.*, 2005).

Manoranjith *et al.* (2012) raised polyclonal antiserum against BSV, which reacted with *Rice tungro bacilliform virus* and *Sugarcane bacilliform virus* (SCBV) in triple antibody sandwich (TAS) ELISA. Tottappilly *et al.* (1998) reported that DAS-ELISA was more effective in detecting BSV antigen than ACP-ELISA. Cherian *et al.* (2003) reported that BSV is moderately immunogenic and polyclonal antiserum is needed for its detection. Ndowora *et al.* (2000) diagnosed BSV by DAS-ELISA and triple antibody sandwich enzyme immunoassay (TAS-ELISA) using polyclonal antibody raised against sugarcane bacilliform virus (SCBV) and BSV isolate from 'Mysore' variety. They reported that TAS-ELISA was more sensitive and effective than DAS-ELISA to detect a wider range of BSV isolates in infected banana.

Dheepa and Paranjothi (2010) serologically diagnosed CMV infecting banana using DAC ELISA.

2.3.1.2 DOT IMMUNOBINDING ASSAY (DIBA)

Rajasulochana *et al.* (2008) reported that Dot-blot-ELISA was relatively more sensitive for the detection of BSV and CMV in banana than ELISA. ELISA and DIBA were found to be most sensitive method than other serological method in detecting BBrMV (Dhanya *et al.*, 2007). Selvarajan (1997) detected BBrMV in infected samples using DIBA technique. Definite blue colour was developed from infected samples, were it was absent in the healthy samples. Abdel - Salam *et al.* (2005) detected BSV infected banana samples using dot and tissue blot immuno assays with antiserum raised against Egyptian isolate of BSV.

2.3.2 MOLECULAR DIAGNOSIS

Since Polymerase chain reaction (PCR) has the power to amplify the target nucleic acid present at an extremely low level and form a complex mixture of heterologous sequences, it has become an attractive technique for the diagnosis of plant virus diseases (Henson and French, 1993). Based on the nucleotide sequence information of several different viruses, specific oligo nucleotide primers can be designed and used in PCR to detect and differentiate viruses at the family, genus, or strain level (Robertson *et al.*, 1991) or for simultaneous detection of unrelated viruses in a sample by using a mixture of virus-specific primer (Nassuath *et al.*, 2000).

Mahadev *et al.* (2013) developed a protocol for early diagnosis of BBTV at tissue culture level itself through optimizing the PCR method by using the viral gene specific primers for coat protein (CP) gene and nuclear shuttle protein (NSP) gene. Galal (2007) amplified genomic BBTV sequence by PCR using DNA isolated from infected banana samples and viruliferous banana aphid. A PCR product of 1000bp was obtained from positive sample. He also observed that the PCR results varied with DNA concentration.

Prakash *et al.* (2010) amplified genomic sequence of BBTV and BSV sequence by PCR using specific primers for BBTV and BSV. Selvarajan *et al.* (2011) standardized protocol of multiplex reverse transcription polymerase chain reaction (mRT – PCR) for the detection of mixed infection due to BBTV and BSV using random primers for the cDNA synthesis.

The primers designed to amplify the DNA of BSV Nigerian isolate amplified DNA of BSV Tamil Nadu isolate producing amplicon of size 644bp in size. The primers used in PCR to amplify BSV did not amplify other *Badnavirus* (Manoranjith *et al.*, 2012). Karanja *et al.* (2010) amplified BSV genome using immune capture PCR (IC-PCR). Mwangi *et al.* (2010) used IC-PCR for BSV amplification. Cherian *et al.*, (2003) amplified the BSV genome with an amplicon of size 730bp. They isolated DNA using DNeasy plant mini kit (Qiagen). Javer *et*

al. (2009) detected BSV particles in different varieties of banana using multiplex IC-PCR. Figueiedo and Brioso (2007) developed protocol for multiplex PCR for detection of BSV and CMV genome. Provost *et al.* (2006) developed multiplex immuno capture polymerase chain reaction (M-IC-PCR) protocol for the detection of episomal BSV genome infecting banana by using *Musa* sequence tagged microsatellite site (STMS) primers and specific primers for BSV.

Prema *et al.* (2012) standardized multiplex PCR for the amplification of DNA from BSV genome and cDNA synthesized from CMV RNA. The annealing temperature 52°C for 30 seconds resulted in the amplification of BSV and CMV genome. BSV genome amplified at 730 bp and CMV genome amplified at 488 bp.

Khan *et al.* (2011) reported that immuno capture reverse transcription PCR (IC-RT-PCR) was more sensitive than DAS-ELISA for the detection of CMV infected banana plant.

Dassanayake (2001) reported that IC-PCR was more labour efficient and less costly than RT-PCR for the detection of BBrMV. Fuxiu *et al.* (2012) developed quadruplex RT-PCR for the simultaneous detection and differentiation of all the four viruses in banana.

2.4 SEQUENCING

The genome of BBTV consist of at least six integral component, each approximately 1Kb size (BBTV DNA-R, -S, -M, -C, -N and –V). DNA-S codes for viral coat protein for encapsidation. DNA-R codes for master replication initiation protein (Karan *et al.*, 1997). BBTV isolates are categorized into two groups, namely the "South Pacific group" and "Asian group" based on the nucleotide sequence identity of DNA-R. The mean sequence difference within each group was 1.9-3 % and between isolates from two groups is 10 % (Karan *et al.*, 1994). Selvarajan *et al.* (2010) reported that the Indian BBTV isolates with distinct geographical origin including Kerala belongs to South Pacific group, except Shervoy and Kodaikanal hill isolate, which neither belongs to South Pacific nor the Asian group. Karan *et al.* (1994) reported that the Indian isolate of

BBTV belongs to South Pacific group.

A high degree of heterogenicity exists among BSV isolate and they differ serologically, genomically and biologically (Geering *et al.*, 2000). Diverse population of BSV was present in West Africa (Agindoton *et al.*, 2006). Harper *et al.* (2004) reported that BSV is highly variable in Uganda. Harper *et al.* (1999) reported that BSV viral sequence may integrate in to the host nuclear genome.

Indian isolates of BBrMV has greater than 96.6% homology with a Phillippines isolate at nucleotide and amino acid level respectively (Rodoni *et al.*, 1997).

Hu *et al.* (1995) divided Cucumber Mosaic Virus strains into two major subgroups, I and II, by serological and molecular methods. Meng Ling *et al.* (1997) identified 2 severe strains (B-CMV-1 and B-CMV-2) and 1 mild strain (B-CMV-3) of banana CMV from Taiwan.

2.5 ELIMINATION OF VIRUS THROUGH MERISTEM CULTURE

Meristem culture offers an efficient method for rapid clonal propagation, production of virus free materials and germplasm preservation. El- Dougdoug *et al.* (2009) observed banana meristem tip of size 0.3 mm is more effective for BBTV, BBrMV eradication in *invitro*. Shirgai *et al.* (2008) eliminated BBTV from infected plant by using meristem culture. Highest number of shoot proliferated with 4 ppm BAP and 1.5 ppm NAA in the variety Amritasagar. There was negative relationship between BAP application and rooting, and positive relationship between NAA application and rooting. Highest number of roots was obtained by NAA at 2mg/L concentration. BBTV was readily transmitted through micropropagation (Thomas *et al.*, 1995). Ali *et al.* 2011 reported that for the *invitro* micopopagation of banana using shoot apical meristem, MS+ BAP (1ppm) gave best response for shoot formation, MS + BAP (1ppm)+ Kinnetin (0.25 ppm) gave best multiplication response, MS + IBA (1ppm) + NAA (0.5ppm) gave best response for rooting. Shoot formation and multiplication response was affected

by temperature.

Due to the wide spread of endogenous para retro virus sequence E (PRVs) of BSV in Musa sp. and inter specific hybrids, BSV has become a main constraint to Musa improvement programme, germplasm exchange and tissue culture (Selvarajan *et al.*, 2011). Tissue culture method resulted in the expression of integrated BSV genome (Lockhart, 1997).

Virus-free plants of banana cvs Poyo and Giant Cavendish infected by *Cucumber mosaic cucumo virus* were produced by meristem tissue culture. Explants were cultured in MS medium supplemented with thiamine HCl (1 mg/litre), pyridoxine (1 mg/litre), nicotinic acid (100 mg/litre) and myo-inositol (100 mg/litre) and solidified with 7 g/litre bacto agar. Virus indexing of plants was done by inoculating cowpea cv. Black test plants. The efficiency of virus elimination was 68.8% for Poyo and 66.8% for Giant Cavendish (Rivas, 1988).

Materials and Methods

3. MATERIALS AND METHODS

The present study was conducted for the diagnosis of BBTV, BBrMV, BSV and CMV infecting banana and production of disease free planting materials of banana variety, Nendran. The experiment was conducted in Department of Plant Pathology, College of Agriculture, Vellayani.

3.1 SYMPTOMATOLOGY

Virus infected banana suckers were collected from the field, they were kept in insect proof green house and expression of symptoms were observed. Infected samples of banana in different cultivars were collected from fields of Kazhakkoottam, Vellayani, Varkala and Adimaly. These samples were subjected to Direct Antigen Coating Enzyme Linked Immuno Sorbent Assay (DAC-ELISA) and Dot Blot Immuno Binding Assay (DIBA) using polyclonal antisera against BBTV, BBrMV, BSV, CMV (Agdia Pvt. Ltd.) and the symptomatology was studied individually and as combined infection.

3.2 HOST PATHOGEN INTERACTION

Physiological changes in banana as a result of four viral infections were studied. The spindle leaf samples were collected from both the infected and healthy three month old Nendran banana plants of Nendran variety and changes in total carbohydrate, chlorophyll, phenol and protein were estimated. The activities of defence related enzymes were also analysed.

3.2.1.1 ESTIMATION OF TOTAL CARBOHYDRATE

Total carbohydrate content was estimated by Anthrone method (Hedge and Hofreiter, 1962). Samples of 100 mg each were weighed out and hydrolyzed with 5 ml of 2.5 N Hydrochloric acid (HCl) in a boiling water bath for three hours. The hydrolyzate was neutralized with solid sodium carbonate until the effervescence ceased. The volume was made up to 100 ml and centrifuged at 5000 rpm for 15

minutes. From the supernatant 0.5 ml aliquot was taken and made up to one ml by adding distilled water. To this 4 ml anthrone reagent was added and heated for eight minutes in a boiling water bath. This was cooled rapidly and absorbance was measured at 630 nm in a spectrophotometer (Systronics UV-VIS Spectrophotometer 118). Amount of carbohydrate present was calculated from standard graph prepared using glucose and expressed in terms of milligrams of glucose equivalent per gram of leaf tissue on fresh weight basis.

3.2.1.2 ESTIMATION OF CHLOROPHYLL

Chlorophyll was estimated by the method described by Arnon (1949). One gram of leaf sample was finely cut and ground in a pestle mortar with 20 ml of 80 per cent acetone. The homogenate was centrifuged at 5000 rpm for five minutes and the supernatant was transferred to a 100 ml volumetric flask. The above procedure was continued till the residue become colourless. The final volume in volumetric flask was made up to 100 ml. Absorbance of the solution at 645, and 663 nm was read in a spectrophotometer against the solvent (80 per cent acetone) as blank. The chlorophyll content was calculated using the following equations and expressed as milligrams chlorophyll per gram tissue.

$$\text{Chlorophyll a} = 12.7(A663)-2.69(A645) \times \frac{V}{1000 \times w}$$

$$\text{Chlorophyll b} = 22.9(A645)-4.68(A663) \times \frac{V}{1000 \times w}$$

$$\text{Total chlorophyll} = 20.2\,(A645 + 8.02\,(A663) \times \frac{V}{1000 \times w}$$

V – Final volume of chlorophyll extract in 80% acetone.

W – Fresh weight of tissue extracted.

3.2.1.3 ESTIMATION OF PROTEIN

Total soluble protein content was estimated as per the procedure described by Bradford (1976). One gram of leaf sample was homogenized in 10 ml, 0.1 M sodium acetate buffer (pH 4.7) (Appendix- I) and centrifuged at 5000 g for 15 minutes at 4° C. The supernatant was used for estimation of soluble protein. The reaction mixture consisted of 0.5 ml enzyme extract, 0.5 ml distilled water and 5 ml of diluted (5 times) dye solution (Appendix II). The absorbance was read at 595 nm in a spectrophotometer against reagent blank. Bovine serum albumin was used as the protein standard. The protein content was expressed as microgram albumin equivalent of soluble protein per gram on fresh weight basis.

3.2.1.4 ESTIMATION OF PHENOL

The phenol content was estimated following the procedure described by Bray and Thorpe (1954). One gram leaf sample was ground in 10 ml of 80 per cent ethanol. The homogenate was, centrifuged at 10000 rpm for 20 min, supernatant was saved and residue was extracted with five times the volume of 80 per cent ethanol and centrifuged. The supernatant was saved and evaporated to dry in a boiling water bath. The residue was dissolved in 5 ml distilled water. An aliquot of 0.3 ml was pipetted out and made up to 3 ml with distilled water. Folin-ciocalteau reagent (0.5 ml) was added and 2 ml of 20 per cent sodium carbonate solution was added to each tube after three minute. This was mixed thoroughly and kept in boiling water for one minute. The reaction mixture was cooled and absorbance was measured at 650 nm against reagent blank. Standard curve was prepared using different concentrations of

catechol and expressed in catechol equivalents as microgram per gram leaf tissue on fresh weight basis.

3.2.2 ESTIMATION OF DEFENCE RELATED ENZYMES

3.2.2.1 ESTIMATION OF PEROXIDASE (PO)

Peroxidase activity was determined according to the procedure described by Srivastava (1987). Leaf sample of 200 mg was homogenized in one ml of 0.1 M sodium phosphate buffer (pH 6.5) (Appendix- III) to which a pinch of polyvinyl pyrrolidone (PVP) was added. The homogenization was done at 4^0C using a mortar and pestle. The homogenate was filtered through a muslin cloth and centrifuged at 5000 rpm for 15 minute at 4^0C. The supernatant was used as the enzyme extract for the assay of PO activity.

The reaction mixture consisting of one ml of 0.05 M pyrogallol and 50 µl of enzyme extract was taken in both reference and sample cuvettes, mixed and kept in a spectrophotometer (Systronics UV-VIS spectrophotometer 118) and the reading was adjusted to zero at 420 nm. The enzyme reaction was started by adding one ml of one per cent hydrogen peroxide (H_2O_2) into sample cuvettes and change in absorbance was measured at 30 seconds interval.

3.2.2.2 ESTIMATION OF POLYPHENOL OXIDASE (PPO)

Polyphenol oxidase activity was determined as per the procedure given by Mayer *et al.* (1965). The enzyme extract was prepared as per the procedure given for the estimation of peroxidase.

The reaction mixture contained one ml of 0.1 M sodium phosphate buffer (pH 6.5) and 50 µl of enzyme extract. The reaction was initiated after adding one ml of 0.01 M catechol. The observations were recorded in a spectrophotometer (Systronics UV-VIS spectrophotometer 118). The change in

absorbance was recorded at 495 nm and PPO activity was expressed as change in the absorbance of the reaction mixture per minute per gram on fresh weight basis.

3.2.2.3 ESTIMATION OF PHENYLALANINE AMMONIA-LYASE (PAL)

PAL activity was analysed based on the procedure described by Dickerson *et al.* (1984). The enzyme extract was prepared by homogenizing one gram leaf sample in 5 ml of 0.1 M sodium borate buffer (pH 8.8) (Appendix- IV) containing a pinch of PVP using chilled mortar and pestle. The homogenate was centrifuged at 10000 rpm for 10 minutes at 4^0C. The supernatant was used for the assay of PAL activity. The reaction mixture contained 3 ml of 0.1 M sodium borate buffer (pH 8.8), 0.2 ml enzyme extract and 0.1 ml of 12 mM L-phenyl alanine prepared in the same buffer. The blank contained 3 ml of 0.1 M sodium borate buffer (pH 8.8) and 0.2 ml enzyme extract. The reaction mixture and blank were incubated at 40^0C for 30 minute and reaction was stopped by adding 0.2 ml of 3N hydrochloric acid. The absorbance was read at 290 nm in a spectrophotometer (Systronics UV-VIS spectrophotometer 118).

PAL activity was expressed as micrograms of cinnamic acid produced per minute per gram on fresh weight basis.

3.2.3.1 SDS- PAGE

Electrophoretic separation of soluble protein of virus infected banana leaves were carried out as per the procedure described by Laemelli (1970).

One gram each of healthy and infected leaf samples were homogenized in 1.5ml of cold denaturing solution (Appendix V) at 4°C. the extract was centrifuged at 5000 rpm for 15 minute. The supernatant was mixed with chilled acetone in the

ratio 1:1 and the protein was allowed to precipitate by keeping the mixture at 4°C for 30 minute. The sample was centrifuged at 5000 rpm for 15 minute at 4°C. The supernatant was removed and the pellet was resuspended in 50 µl of denaturing solution and vortexed. The homogenate was centrifuged at 5000 rpm for 15 minutes. The supernatant was mixed with 10 µl of sample buffer and kept in a boiling water bath for 3 minutes. These samples were used subjected to electrophoresis using SDS - PAGE. The protein concentration was adjusted in each sample to strength of 100 µg of protein following Bradford method.

Reagents

a) Acrylamide stock (30 %)

Acrylamide	–	29.2 g
Bis-acrylamide	–	0.8 g
Double distilled water	–	100.0 ml

b) Separating (resolving) gel buffer stock (1.5 M Tris-HCl, pH 8.8)

Tris base (18.15 g) was dissolved in approximately 50 ml of double distilled water. The pH was adjusted to 8.8 with 6 N HCl and made up the volume to 100 ml with double distilled water and stored at 4°C.

c) Stacking gel buffer stock (0.5 M Tris-HCl pH 6.8)

Tris base (6.0 g) was dissolved in approximately 60 ml of double distilled water and adjusted the pH to 6.8 with 6 N HCl and the volume was made up to 100 ml with double distilled water and stored at 4°C.

d) Polymerising agents

Ammonium persulphate (APS) 10 per cent prepared freshly before use.

TEMED – Fresh from refrigeration.

e) Electrode buffer pH 8.3

Tris base	-	6.0 g
Glycine	-	28.8 g
SDS	-	2.0 g
Double distilled water	-	2 litre

f) Sample buffer

Double distilled water	-	2.6 ml
0.5 M Tris HCl pH 6.8	-	1.0 ml
2-mecaptoethanol	-	0.8 ml
Glycerol	-	1.6 ml
SDS 20 % (w/v)	-	1.6 ml
0.5 % Bromophenol blue	-	0.4 ml

g) Staining solution

Coomassie brilliant blue R 250	-	0.1 g
Methanol	-	40.0 ml
Glacial acetic acid	-	10.0 ml
Double distilled water	-	50.0 ml

h) Destaining solution

As above without Coomassie brilliant blue R 250

Procedure

Separating gel was first casted followed by stacking gel by mixing the various solutions as indicated below.

a) Preparation of separating gel (12%)

Double distilled water	-	6.7 ml
Tris HCl, pH 8.8	-	5.0 ml
SDS 10 %	-	0.2 ml
Acrylamide stock	-	8.0 ml

The above solution was mixed well and degassed for three minutes and then the following were added immediately.

Freshly prepared 10 % Ammonium persulphate (APS)	-	0.10 ml
Tetra methyl ethylenediamine (TEMED)	-	0.01 ml

The separating gel was mixed well and poured immediately between glass plates and a layer of water was added above the polymerizing solution to quicken the polymerization process.

b) Preparation of stacking gel

Double distilled water	-	6.1 ml
Tris HCl, pH 6.8	-	2.5 ml
SDS 10 %	-	0.2 ml
Acrylamide stock	-	1.3 ml

The solution was mixed well, degassed and the following were added.

APS 10 %	-	0.05 ml
TEMED	-	0.1 ml

The water layered over the separating gel was removed and washed with a little electrode buffer and then the stacking gel was poured over the polymerized separating gel, after keeping the comb in position.

After polymerization, the comb was removed and the samples were loaded into the wells. Standards with known molecular weight was also added to one well. The electrophoresis was performed at 100 V till the dye reached the separating gel. Then the voltage was increased in 200 V and continued till the dye reached the bottom of the gel. Immediately after electrophoresis the gel was removed from the glass plates and incubated in the staining solution for overnight with uniform shaking. Then the gel was transferred to the destaining solution. The protein appeared as bands and the gel was photographed after placing it on a transilluminator (Appligene Model White / UV TMW-20).

3.2.3.2 ELECTROPHORETIC ANALYSIS OF ISOZYME

Electrophoresis of protein extracts from plant tissues using different kinds of support media and buffer systems (Appendix VI) allows separation of the multiple forms of enzymes (isozymes) on the basis of charge and molecular size.

The present work was undertaken to study the enzyme alterations in healthy as well as virus infected banana leaves.

Enzyme extraction and assay of peroxidase

Soluble and ionically bound enzymes were extracted by grinding the sample under chilled condition in 50 mM Tris-HCl (pH 7.6) in the ratio of 1:2 w/v. The homogenate was centrifuged at 15,000 rpm for 10 minutes at 4°C. The resulting supernatant was used for isozyme analysis. The protein content was adjusted in each sample to the strength of 100 µg of protein following Bradford method (Bradford, 1976).

Isozyme separation and staining

Discontinuous anionic polyacrylamide gel electrophoresis was conducted under non-dissociating conditions as described by Wagih and Coutts (1982) with slight

modification. Proteins extracted by 50 mM Tris (pH 7.6) were separated by gel electrophoresis in 7.5 per cent gel. The gel was prepared using the stock solution prepared for protein gel electrophoresis with out SDS (native gel). 2% Triton X - 100 was added in place of SDS.

Reagents

a) Separating gel (7.5 %)

Tris chloride buffer stock solution (pH 8.9)	- 5 ml
Resolving gel acrylamide solution	- 10 ml
Triton X 100	- 2%
Distilled water	- 25 ml
APS	- 300 µl

b) Stacking gel (4%)

Tris chloride buffer stock, pH 6.7	- 2.5 ml
Resolving gel acrylamide solution	- 3.1 ml
Triton X 100	- 2%
Distilled water	- 14.1 ml
APS	- 300 µl

For peroxidase enzyme, the gel was incubated in 0.6 M sodium acetate buffer (pH 5.4) containing 0.5 per cent O dianisidine HCl for 30 minutes at room temperature. The gel was transferred to 0.1 M hydrogen peroxide until visible bands were developed.

3.3 IMMUNOLOGICAL AND MOLECULAR DIAGNOSIS

3.3.1 IMMUNOLOGICAL DETECTION

3.3.1.1 DIRECT ANTIGEN COATING – ENZYME LINKED IMMUNOSORBENT ASSAY (DAC- ELISA)

The detection of BBTV, BBrMV, BSV and CMV was done using DAC-ELISA. Diseased samples were taken from the fields in Vellayani, Kazhakoottam, Adimaly. Treatments were fixed in such a way that each sample was tested for four viruses.

Procedure

One gram of infected young leaf was homogenized in 5 ml of coating buffer (carbonate buffer) containing two per cent (w/v) PVP under chilled condition. Healthy plant extract was prepared by using leaves of healthy plants.

The homogenate was centrifuged at 5000 rpm for 10 minutes at 4°C. Samples were dispensed at the rate of 100 µl into Nunc immunological plates. The treatments were replicated thrice for each sample. After incubation for two hours at 37°C, the wells were washed with phosphate buffer saline- tween (PBS-T) three times each for duration of three minutes using a plate washer (PW-40, BIORAD). Blocking was done with 100 µl of one per cent bovine serum albumin (BSA) for 60 minutes at 37°C. After incubation blocking agent was removed, plates were washed with PBS-T as before. 100 µl polyclonal antibody (200 : 1 dilution, Agdia Pvt. Ltd.) of BBTV, BBrMV, BSV and CMV diluted in PBS-T poly vinyl pyrrolidone (PBS-TPO) were loaded to the well as per the treatment and incubated overnight at 4°C. The plates were washed with PBS-T as before and treated with 100µl of alkaline phosphatase conjugated anti-rabbit immunoglobulin antiserum (1: 10000 dilutions, SIGMA – Aldrich) diluted in PBS-TPO and incubated for 2 h at 37°C. Wells were washed with PBS-T as before. The substrate para -nitro phenyl phosphate (*P*-NPP) in diethanol

amine buffer (1 mg/ml) was added to each well (100µl/well) and incubated for 30 minutes at 37°C. Reaction was stopped by adding 50 µl of 4 % sodium hydroxide. The absorbance was read at 405 nm in an ELISA reader (BIORAD, Model 680 Microplate reader). (Appendix VII).

3.3.1.2 DOT IMMUNO BINDING ASSAY (DIBA) FOR THE DETECTION OF BANANA VIRUSES

DIBA was carried out to detect the presence of BBTV, BBrMV, BSV and CMV in infected banana leaves.

Procedure

- Tissue was extracted in antigen extraction buffer (1:10 w v) and filtered through cheese cloth
- 0.8 ml of expressed sap was taken in an 1.5ml eppendorf tube to which 0.4 ml chloroform was added
- The mixture was vortexed and centrifuged at 12,000 g for two minutes
- The clarified sap (upper aqueous layer) was mixed with antigen extraction buffer (1:4 ratio) and vortexed
- Nitrocellulose membrane (NCM) in squares of 1 x 1 cm was drawn with pencil and floated in Tris Buffer Saline (TBS) and air dried
- 10 µl of the sample was spotted to the centre of each square and allowed it to dry
- Treated NCM was immersed in blocking solution with gentle oscillation for one hour at room temperature
- NCM was rinsed in TBS for 10 minutes and incubated overnight at 4°C in crude antiserum diluted in TBS-spray dried milk (SDM).

- NCM was again rinsed in TBS for 10 minutes and incubated for one hour at room temperature in secondary antibody (antirabbit IgG alkaline phosphatase conjugate diluted in TBS-SDM).

After rinsing in TBS for 10 minutes, NCM was incubated in substrate solution (a solution of nitro blue terazolium and bromo chloro indolyl phosphate) at room temperature in the dark for the colour development. NCM was rinsed in fixing solution for 10 minutes and then air dried between Whatman filter paper sheets and stored. (Appendix VIII).

3.3.2 MOLECULAR DIAGNOSIS

3.3.2 MOLECULAR DIAGNOSIS

Molecular diagnosis using polymerase chain reaction (PCR) was performed for the detection of BBTV, BBrMV, BSV and CMV. DNA was extracted to diagnose the presence of BBTV and BSV. RNA was extracted to diagnose the presence of CMV and BBrMV. DNA isolation was done using DNA easy plant mini kit (QIAGEN: Cat: No: 69104). RNA isolation was done using the protocol developed by Salzman *et al.* (1999).

RNA isolation of BBrMV and CMV

Protocol

1. Grinded 100 mg tissue to fine powder with liquid nitrogen using a pestle and mortar.
2. Added powdered tissue to two ml eppendorf tube containing one ml extraction buffer. Shaken vigorously for 1 minute
3. Added equal volume of chloroform : isoamyl alcohol (24:1) mixture. Shaken vigorously for 20 minute.
4. Centrifuged it at 16,000 g for 10 minute at 4 ^{o}C to separate phase.

5. The upper phase was carefully removed to another 2 ml centrifuge tube and repeated above 2 steps until no noticeable interphase material was evident.
6. Removed the supernatant to a clean 2 ml eppendorf tube, added 2X homogenate volume of absolute ethanol and 0.1 X homogenate volumes of 5M NaCl. Precipitated it at -20 °C for overnight.
7. Centrifuged it at 16,000 g for 10 minute at 4 °C. Resuspented the pellet in 1ml di ethyl polycarbonate (DEPC) treated water. Centrifuged it at 20,000g for 5 minutes and recovered the supernatant.
8. Added an equal volume of TE saturated phenol (pH 8) : chloroform: isoamyl alcohol (25:24:1) and shaken it for 5-10 minutes at room temperature to separate phases.
9. Carefully transferred upper phase to a clean tube, avoiding any interphase material. Repeated the above step two more times, until there was no appreciable accumulation of material visible at interphase after centrifugation.
10. Added 2X volume of absolute ethanol and 0.1 X volume of 5 M NaCl. Precipitated it at -20 °C for overnight.
11. Centrifuged it at 16,000 g for 15 minutes at 4 °C and resuspended the pellet in 0.5 ml di ethyl polycarbonate (DEPC) - treated distilled water.
12. Made sample volume to 1ml with DEPC treated water and added 0.33ml of 8M LiCl. Precipitated it at -20 °C for overnight.
13. Centrifuged at 12,000 g for 20minutes at 4 °C. Wash pellet with 0.4 ml 80% ethanol. Allowed the pellet to dry. Resuspended with 0.1 ml DEPC treated water.

c-DNA synthesis of BBrMV RNA and CMV RNA

cDNA was synthesized using AMV- RT- PCR kit (GeNei, Cat No: 6106624000011730). Reverse transcription was performed at 42 °C for one hour followed by 94 °C for 2 minutes in a PCR thermocycler (BIORAD).

3.3.2.1 PCR protocol for detection of BBTV

PCR Analysis

PCR amplification reactions were carried out in a 20 µl reaction volume which contained 2 µl of Taq buffer (contains 15 mM $MgCl_2$) (Genei), 0.4 µl of 10mM dNTP mix (Genei), ~20ng DNA, 5 µl of Taq DNA polymerase enzyme (Genei), 1µl 0f 5pM forward and reverse primers (SIGMA-ALDRICH).

The PCR amplification using CP gene and NSP gene of BBTV was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

Primers used

Target	Primer Name	Direction	Sequence (5′ → 3′)	Reference
CP	CP-F1	Forward	ATGGCTAGGTATCCGAAG	(Mahadev *et al.*, 2013)
	CP-R1	Reverse	CCAGAACTACAATAGAATGCC	
NSP	NSP-F1	Forward	CCTCGCAAGGTACTTCTTAG	(Mahadev *et al.*, 2013)
	NSP-R1	Reverse	CCATGTCTCTGCTCCAATCT	

PCR amplification programme for the primer pair CP F1 & CP R1

94 °C	-	5 min	
94 °C	-	1 min	} 35 cycles
56 °C	-	1 min	
72 °C	-	90 sec	
72 °C	-	10 min	
4 °C	-	∞	

PCR amplification programme for the primer pair NSP F1 & NSPR1

94 °C	-	5 min	
94 °C	-	1 min	} 35 cycles
51°C	-	1 min	
72 °C	-	90 sec	
72 °C	-	10 min	
4 °C	-	∞	

3.3.2.2 PCR protocol for detection of BSV

PCR Analysis

PCR amplification reactions were carried out in a 20 µl reaction volume which contained 2 µl of Taq buffer (contains 15 mM $MgCl_2$) (Genei), 0.4 µl of 10 mM dNTP mix (Genei), ~20ng DNA, 5 µl of Taq DNA polymerase enzyme (Genei), 1µl 0f 5pM forward and reverse primers (SIGMA-ALDRICH).

The PCR amplification using CP gene of BSV was carried out in a PCR thermal cycler (*GeneAmp PCR System 9700, Applied Biosystems*).

Primers used

Target	Primer Name	Direction	Sequence (5′ → 3′)	Reference
CP	BSV 4673	Forward	GGAATGAAAGAGCAGGCC	(Manoranjitham *et al.*, 2012)
	BSV 5317	Reverse	AGTCATTGGGTYCAACCTCTGTCCC	
CP	BSV F	Forward	AGAGTGGGTTTCATCAAGTAGC	(Cherian *et al;* 2004)
	BSV R	Reverse	GAATTCCCGCTCGCCAAAG	

PCR amplification programme for the primer pair BSV 4673 & BSV 5317

98 °C	-	30 sec	
98 °C	-	5 sec	} 40 cycles
55 °C	-	10 sec	
72 °C	-	15 sec	
72 °C	-	1.00 min	
4 °C	-	∞	

PCR amplification programme for the primer pair BSV F & BSV R

94 °C	-	4 min	
94 °C	-	30 sec	} 30 cycles
58 °C	-	30 sec	
72 °C	-	30 sec	
72 °C	-	10 min	
4 °C	-	∞	

3.3.2.3 PCR protocol for detection of CMV

PCR Analysis

PCR amplification reactions were carried out in a 25 µl reaction volume which contained 2.5 µl of Taq buffer (contains 15 mM $MgCl_2$) (Genei), 0.5 µl of 10mM dNTP mix (Genei), 1 µl cDNA, 6.25 µl of 3 unit Taq DNA polymerase enzyme (Genei), 1µl 0f 0.2 µM forward and reverse primers (SIGMA-ALDRICH). The PCR amplification using CP genes of CMV was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

Primers used

Target	Primer Name	Direction	Sequence (5′→ 3′)	Reference
CP	CMV.F	Forward	ATGGACAAATCTGAATCAACC	(Khan *et al.*, 2011)
	CMV.R	Reverse	TCAAACTGGGAGCACCCC	

PCR amplification programme for the primer pair CMV F & CMV R

95 °C -	5 min		
95 °C -	1 min	}	30 cycles
57.8 °C -	1 min		
72 °C -	1 min		
72 °C -	7 min		
4 °C -	∞		

3.3.2.4 PCR protocol for detection of BBrMV

PCR Analysis

PCR amplification reactions were carried out in a 25 µl reaction volume which contained 2.5 µl of Taq buffer (contains 15 mM $MgCl_2$) (Genei). 0.5 µl of 10mM dNTP mix (Genei). 1 µl cDNA. 6.25 µl of 3 unit Taq DNA polymerase enzyme (Genei). 1µl 0f 0.2 µM forward and reverse primers (SIGMA-ALDRICH).

The PCR amplification using bract 1 and bract2 was carried out in a PCR thermal cycler (Gene Amp PCR System 9700, Applied Biosystems). Different annealing temperatures were checked (46.9 °C , 48.9 °C, 51.2 °C , 52.9 °C , 54 °C, 54.2 °C, 55 °C, 57 °C, 59 °C, 64 °C,). The PCR amplification using BBrMV F and BBrMV R was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). Different annealing temperatures were checked (50 °C , 51 °C , 52 °C , 54 °C , 56.4 °C, 56 °C, 54.8 °C, 59 °C).

Primers used

Target	Primer Name	Direction	Sequence (5′ → 3′)	Reference
CP	Bract 1	Forward	GACATACCAAATTTGAATGGCACATGG	(Rodoni *et al.*, 1997)
	Bract 2	Reverse	CCATTATCACTCGATCAATACCTCACG	
CP	BBMV F	Forward	AACGCTCAGCCTACTTTCG	(Dassanayake *et al.*, 2001)
	BBMV R	Reverse	CATATCACGCTTCACATCTTCA	

PCR amplification programme for the primer pair bract 1 & bract 2

94 °C	-	5 min	
94 °C	-	20 sec	} 30 cycles
Varied annealing temperature	-	20 sec	
72 °C	-	20 sec	
72 °C	-	10 min	
4 °C	-	∞	

PCR amplification programme for the primer pair BBrMV F & BBrMV R)

94 °C	-	2 min	
94 °C	-	30 sec	} 35 cycles
Varied annealing temperature	-	30 se	
68 °C	-	45 sec	
68 °C	-	7 min	
4 °C	-	∞	

3.3.2.4 Agarose gel electrophoresis of PCR products

The PCR products were checked in 1.3% agarose gels prepared in 1X TBE buffer containing 0.5 µg/ml ethidium bromide. 1 µl of 6X loading dye was mixed with 7 µl of PCR products and was loaded and electrophoresis was performed at 60V power supply with 0.5X TBE as electrophoresis buffer for about 1 hour, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was a 2-log DNA ladder (NEB) and 1 kb DNA ladder (Gene ruler). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

3.3.2.5 ExoSAP-IT Treatment

ExoSAP-IT (GE Healthcare) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications.

Five micro litres of PCR product was mixed with 2 µl of ExoSAP-IT and incubated at 37°C for 30 minutes followed by enzyme inactivation at 80°C for 15 minutes.

3. 4 Sequencing of the PCR products

3.4.1 Sequencing using BigDye Terminator v3.1

Sequencing was done at the Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram. Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems , USA), following manufactures protocol.

The PCR mix consisted of the following components:

PCR Product (ExoSAP treated) - 10-20 ng

Primer	-	3.2 pM
Sequencing Mix	-	0.28 µl
DMSO	-	0.30 µl
5x Reaction buffer	-	1.86 µl
Sterile distilled water	-	make up to 10µl

The sequencing PCR temperature profile consisted of a 1st cycle at 96°C for 2 minutes followed by 30 cycles at 96°C for 30 sec, 50°C for 40 sec and 60°C for 4 minutes.

3.4.2 Post Sequencing PCR Clean up

1. Master mix I of 10 µl milli Q and 2 µl 125 mM EDTA per reaction and master mix II of 2 µl of 3M sodium acetate pH 4.6 and 50 µl of ethanol were prepared.
2. 12 µl of master mix I was added to each reaction containing 10 µl of reaction contents and was properly mixed.
3. 52 µl of master mix II was added to each reaction.
4. Contents were mixed by inverting and incubated at room temperature for 30 minutes
5. Spun at 14,000 rpm for 30 minutes
6. Decanted the supernatant and added 100 µl of 70% ethanol
7. Spun at 14,000 rpm for 20 minutes.
8. Decanted the supernatant and repeated 70% ethanol wash
9. Decanted the supernatant and air dried the pellet.

The cleaned up air dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems).

3.4.3 Sequence Analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Drummond *et al.*, 2010).

The gene sequence of the BBTV, BSV, CMV were analysed using BLAST for finding out the sequence similarity with sequences available in Genebank. The sequences obtained in the BLAST output were aligned using Clustal X 1.81 and the cluster dendrogram was constructed using PHYLIP package.

3.5 MERISTEM CULTURE

3.5.1 Source of explants

Infected banana suckers collected from field were used for the study. The nutrient medium developed by Murashige and Skoog (1962) was used as the basal medium (Appendix IX).

Stock solution of macro, micro minor nutrients, iron and vitamins were prepared by Murashige and Skoog (1962). Stock solution of growth regulator benzyl amino purine (BAP) and (IAA) was prepared.

3.5.2 Preparation of sterile tissue

The meristem portion separated from infected suckers was thoroughly washed in tap water and was cut in to handy sizes. These meristems were soaked in two percent labolene solution for ten minutes and again washed with distilled water for three times to remove any trace of solution. The meristem was transferred to the bavistin (0.1%) for 30 minutes. Then the meristem was washed in distilled water for three times. The meristem was treated with 0.08% mercuric chloride for 10 minutes in laminar air flow chamber. The treated portions were washed in sterile distilled water for three times.

3.5.3 Dissection and explants preparation

Using sterile forceps and blade the disinfected meristem were cut in to different sizes 5mm to 2cm.

3.5.3 Inoculation of explants

The meristem explants were inoculated in the jam bottle having medium (Murashige and Skoog media (MS) + CuSO4 15ppm + charcoal 500mg per litre) in such a way that one third of the explants were inserted in to the media.

3.5.4 Incubation of cultures

Following excision and inoculation. the cultures were placed in dark for 24 hrs and then kept in incubation room at 24 ± 4 °C giving photoperiod of 16h.

3.5.5 Induction media

MS media with BAP (4PPM) and NAA (1.5ppm) used for induction.

3.5.6 Virus indexing of issue culture plants

Virus indexing of tissue culture plants was performed using DAC-ELISA and PCR.

Results

4. RESULTS

4.1 SYMPTOMATOLOGY

Symptoms of BBTD were observed in the cultivar Nendran, Palayankodan and Robusta. All the cultivars produced similar symptom. The expressions of symptoms due to BBTV infection were observed in all the growth stages of the plant.

The infected leaves were observed to be small, brittle with thickened veins and remained bunched at the top of the psuedostem (Plate 1). The infected spindle leaves were smaller than older leaves (Plate 2). Plants with later infection produced bunches with reduced size, weight and misshapen fingers (Plate 3)

The suckers from infected mother plant were observed to be short with bunched leaves and reduced spindle leaf (Plate 4). The infected suckers did not produced fruits.

Symptoms of BBrMD were observed in the cultivar of Nendran, Njalipoovan, Palayankodan, Red banana and Matti. The characteristic reddish spindle shaped lesion were observed in the pseudostem (Plate 5), bunch stalk (Plate 6), leaf petiole (Plate 7), and bract (Plate 8). As the disease progress, the reddish spindle shaped lesion was observed to be necrotic (Plate 9).

Leaves of infected plants showed characteristic chlorotic spindle shaped lesion on the leaf lamina (Plate 10). The chlorotic spots were easily distinguished when the infected leaf was held against light. Purple coloured spindle shaped lesion were also observed in the undersurface of the leaf lamina (Plate 11).

The infected plant gave low yield than healthy plant (Plate 12). Some infected plants produced travellers palm like appearance (Plate 13). The suckers from infected mother plant also produced characteristic reddish spindle shaped lesion (Plate 14).

The characteristic reddish coloured spindle shaped lesion was not observed in the petiole of the cultivar Matti.

The symptoms of BSV appeared mainly on the leaves. Leaves of infected plant showed chlorotic streaks parallel to the veins (Plate15). Later the chlorotic streaks became necrotic (Plate 16). Some BSV infected plans showed vertical striation on the leaf lamina (Plate 17).

The symptoms of CMV appeared mainly on the leaves. Initial symptom includes inter veinal chlorotic streaks in the leaf lamina (Plate 18), which appeared as symptom of BSV. Later the streaks enlarged and formed mosaic regions (Plate 19). Initially the mosaic symptoms appeared to be localized, later the mosaic symptom spread on entire leaf lamina (Plate 20). The necrosis of the spindle leaf was also observed (Plate 21).

In case of combined infection virus individually produced their characteristic symptoms.

Plate 1. Bunching of leaves.

Plate 2. Reduced spindle leaf.

Plate 3. BBTV infected Robusta variety showing reduction in bunch size

Plate 4 Suckers from BBTV infected mother plant.

Plate 5. Reddish spindle shaped lesions in the psuedostem

Plate 6. Reddish spindle shaped lesion in the bunch stalk due to BBrMV infection.

Plate 7. Reddish spindle shaped lesion in the petiole infected with BBrMV

Plate 8. Reddish spindle shaped lesion in the bract of BBrMV infected banana

Plate 9. Necrotic spindle shaped lesion in the midrib caused by BBrMV

Plate 10. Chlorotic spindle shaped lesion in the leaf lamina

Plate 11. Purple coloured spindle shaped lesion on the leaf margin due to BBrMV infection.

Plate 12. Reduction in size of bunch due to BBrMV infection

Plate 13. Travellers Palm like phyllotaxy due to BBrMV infection

Plate 14. Suckers from infected mother plant showing severe symptoms of BBrMV infection

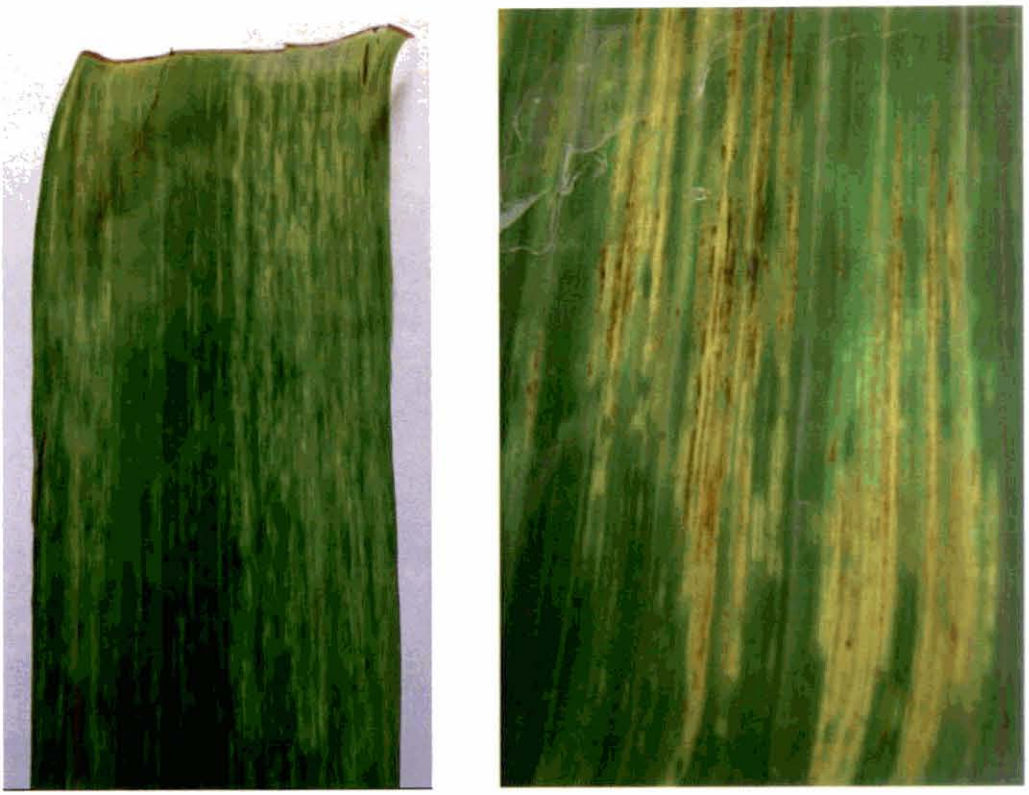


Plate 15. Chlorotic streaks parallel to the vein due to BSV infection.

Plate 16. Necrotic srteaks on the
leaf lamina due to BSV infection

Plate 17. Vertical striation on the leaf lamina due to BSV infection.

Plate 18. Interveinal chlorotic streaks in the leaf lamina due to CMV infection

Plate 19 a. Mosaic symptom on the leaf lamina due to CMV infection

Plate 19 b. Mosaic symptom on the leaf lamina due to CMV infection

Plate 20a. Mosaic pattern on entire leaf lamina due to CMV infection

Plate 20 b. Mosaic pattern on entire leaf lamina due to CMV infection

Plate 21 a. Necrosis of the spindle leaf due to CMV infection

Plate 21b. Necrosis of unfurled spindle leaf due to CMV infection.

4.2 HOST PATHOGEN INTERACTION

4.2.1.1 ESTIMATION OF TOTAL CARBOHYDRATE

The total carbohydrate content of healthy and infected banana plants was estimated and the results are presented in the Table 1.There was significant difference between total carbohydrate in healthy and infected plants. The total carbohydrate content of BBTV, BBrMV, and BSV infected banana plants were less than that of healthy plant. The maximum reduction in total carbohydrate was shown by BBTV infected plants (46 %). The total carbohydrate content of CMV infected plants was more than that of healthy (134 %).

Table 1. Changes in total carbohydrate content (mg/ g leaf tissue) in the spindle leaves of banana plans in response to viral infection.

Treatment	*Carbohydrate content (mg g)	Per cent increase (+) or decrease (-) over healthy
Healthy	79	-
BBTV	43	-46
BBrMV	75	-5
BSV	67	-15
CMV	185	+ 134

*Mean of three replication

CD Values : Healthy vs Infected: 5.313928

4.2.1.2 ESTIMATION OF CHLOROPHYLL

Chlorophyll was estimated by method described by Arnon (1949) and the results are presented in the Table 2 .There was a significant difference in total chlorophyll, chlorophyll a, chlorophyll b content in between infected samples and healthy plants.

The maximum reduction in total chlorophyll was shown by CMV infected plants (71 %). The least reduction in total chlorophyll content was shown by

BBrMV infected plants (58%). The reduction in chlorophyll content showed by BBTV & BSV was 70%.

The maximum reduction in chlorophyll a (79 %) was shown by CMV infected plant where chlorophyll b (74%) was shown by CMV and BSV infected samples. The least reduction in chlorophyll a (57%) and chlorophyll b (42%) was shown by BBrMV infected plants. The reduction in chlorophyll a content showed by BBTV & BSV was 68%. The reduction in chlorophyll b content showed by BBTV was 63%.

Table 2. Changes in chlorophyll content (mg/g leaf tissue) in the spindle leaves of banana plants in response to viral infection.

Treatment	*Chlorophyll a	Per cent decrease over healthy	*Chlorophyll b	Per cent decrease over healthy	*Total chlorophyll	Per cent decrease over healthy
Healthy	0.47	-	0.19	-	0.66	-
BBrMV	0.2	-57	0.11	-42	0.28	-58
BBTV	0.15	-68	0.07	-63	0.2	-70
BSV	0.15	-68	0.05	-74	0.20	-70
CMV	0.1	-79	0.05	-74	0.19	-71

*Mean of three replication

CD Values	Total chlorophyll	Chlorophyll a	Chlorophyll b
Healthy vs Infected	2.152356	7.617306	1.150523

4.2.1.3 ESTIMATION OF PHENOL

The total phenol content was estimated according to the procedure given by Bray and Thorpe (1954) and the results are presented in the Table 3. There was a significant difference between phenol content between healthy and infected plants.

Enhanced level of phenol content was observed in infected samples when compared to healthy samples. When compared infected sample showed percentage increase of 76%, 36%, 33% and 75% for BBTV, BBrMV, BSV and CMV respectively over healthy samples.

Table 3. Changes in total phenolic content (mg/ g leaf tissue) in the spindle leaves of banana plants in response to viral infection.

Treatment	Phenol content (mg/g)	Per cent increase (+) or decrease (-) over healthy
Healthy	0.75	-
BBTV	1.32	+76
BBrMV	1.02	+36
BSV	1	+33
CMV	1.31	+ 75

*Mean of three replication

CD Value : Healthy vs. Infected : 0 .1409127

4.2.1.4 ESTIMATION OF PROTEIN

Estimation of protein was carried out as per the procedure given by Bradford (1976) and the results are presented in the Table 4.The result indicated that the infected samples have higher protein content than healthy samples. The highest protein content was shown by BBTV (150%) infected sample followed by BBrMV (75%), BSV (67%) and CMV (50%).

Table 4. Changes in total protein content (mg/ g leaf tissue) in the spindle leaves of banana plants in response to viral infection.

Treatment	Protein content (mg/g)	Per cent increase (+) or decrease (-) over healthy
Healthy	0.12	-
BBTV	0.3	+150
BBrMV	0.21	+75
BSV	0.2	+67
CMV	0.18	+ 50

*Mean of three replication

CD Value. Healthy vs Infected : 7.107911

4.2.2 DEFENCE RELATED ENZYMES

4.2.2.1 PEROXIDASE

There was a significance difference in peroxidase activity between healthy and infected sample (Table 5). The per cent increase over healthy was maximum in BBTV infected sample (892%) and minimum in CMV infected sample (100%). The percentage increase for BBrMV and BSV were 561% and 623% respectively.

Table 5. Effect of virus infection on peroxidase activity per minute per gram tissue of banana plants

Treatment	Change in OD value per gram per minute	Per cent increase (+) or decrease (-) over healthy
Healthy	0.65	-
BBTV	6.42	+892
BBrMV	4.3	+561
BSV	4.7	+623
CMV	1.3	+ 100

*Mean of three replication

CD Value : Healthy vs Infected : 2.324677

4.2.2.2 POLYPHENOL OXIDASE

Compared to healthy samples there was an increase in polyphenol oxidase activity in all virus infected sample (Table 6). The per cent increase over healthy was maximum in BBrMV infected sample (70%) and minimum in CMV infected sample (1.2%). The percentage increase for BBTV and BSV were 44% and 48% respectively.

Table 6. Effect of virus infection on polyphenol oxidase activity per minute per gram tissue of banana plants

Treatment	Change in OD value per gram per minute	Per cent increase (+) or decrease (-) over healthy
Healthy	3.12	-
BBTV	4.5	+44
BBrMV	5.3	+70
BSV	4.6	+48
CMV	3.16	+ 1.2

*Mean of three replication

CD Value : Healthy vs Infected : 2.939287

4.2.2.3 PHENYL ALANINE AMMONIA-LYASE

Compared to healthy samples there was an increase in PAL activity in all virus infected sample (Table 7). The percent increase over healthy was maximum in CMV infected sample (4.8%) and minimum in BBrMV infected sample (0.19%). The percentage increase for BBTV and BSV were 1.2% and 3.9% respectively.

Table 7. Effect of virus infection on PAL activity per minute per gram tissue of banana plants.

Treatment	*Change in OD value per µg per gram per minute	Per cent increase (+) or decrease (-) over healthy
Healthy	11.7	-
BBTV	26	+1.2
BBrMV	14	+0.19
BSV	57	+3.9
CMV	68	+ 4.8

*Mean of three replication

CD Value : Healthy vs Infected : 1.050524

4.2.3.1 SDS-PAG (SODIUM DODECYL POLYACRYLAMIDE GEL ELECTROPHORESIS

Protein profile of healthy and infected banana samples due to BBTV, BBrMV, BSV and CMV were analysed using SDS-PAGE. The virus infected sample showed extra band in their protein profile. The molecular weight of the extra band was estimated using protein markers loaded along with the samples.

The protein profile of BBTV infected sample showed one extra band with molecular weight of approximately 20 kDa (Plate 22).

The protein profile of BBrMV infected sample showed two major protein band with molecular weight of 38 kDa and 29 kDa and one minor protein band with molecular weight of 22 kDa.(Plate 23).

The protein profile of BSV infected sample showed three extra protein bands. The molecular weights of these additional proteins were 25 kDa, 19 kDa, and 12 kDa (Plate 24).

The protein profile of CMV infected sample showed one extra band with molecular weight of 25 kDa (Plate 25).

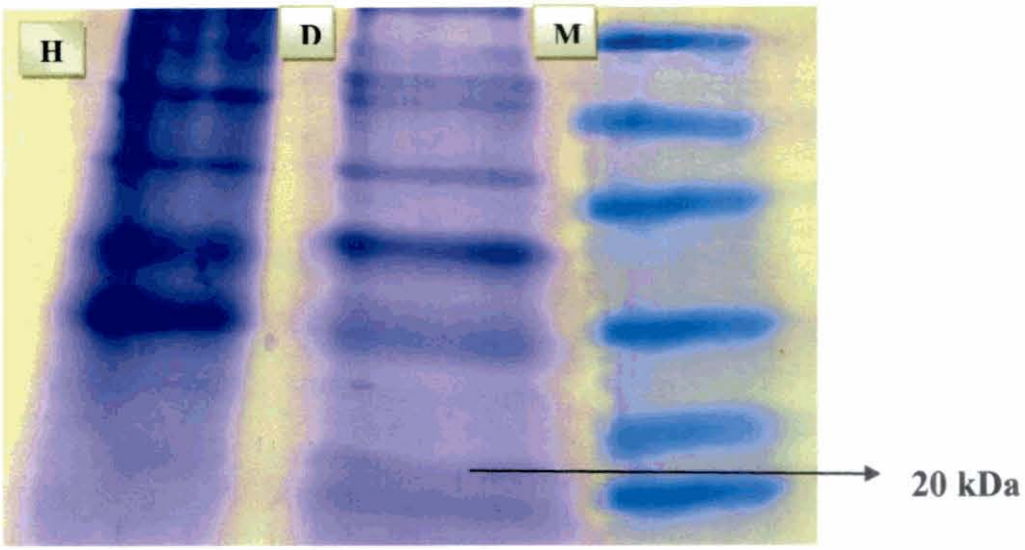


Plate 22. SDS PAGE analysis of healthy and BBTV infected samples

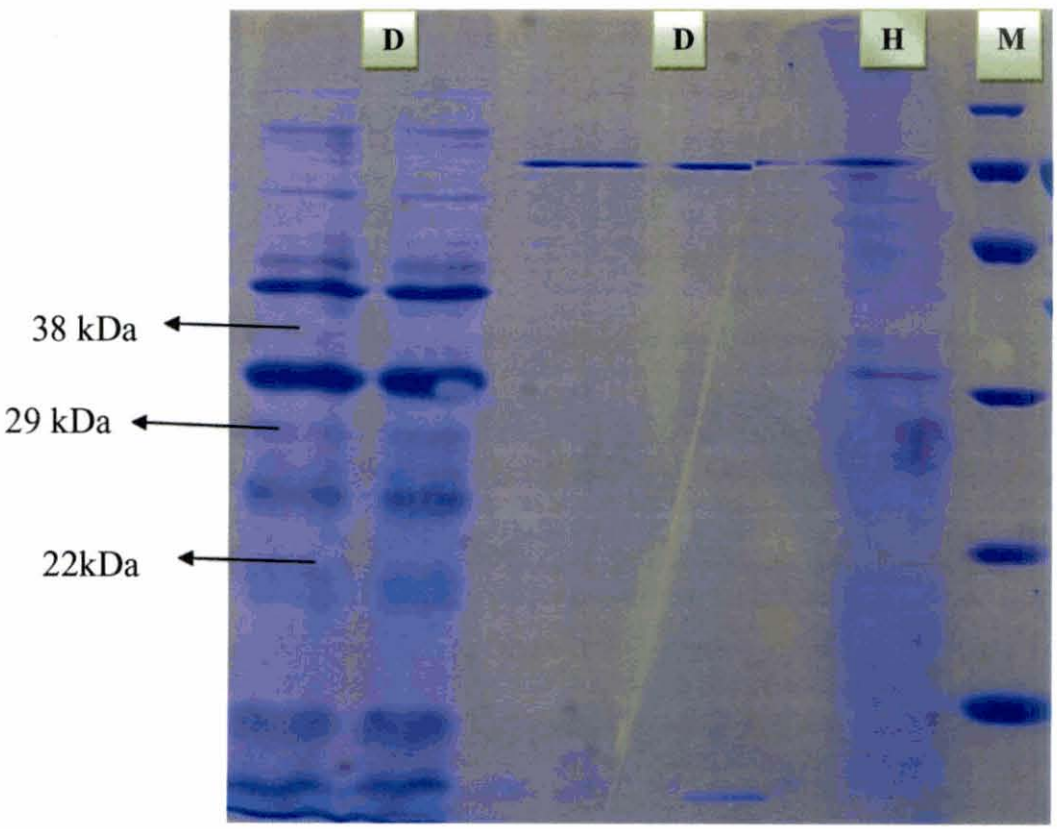


Plate 23. SDS PAGE analysis of healthy and BBrMV infected samples

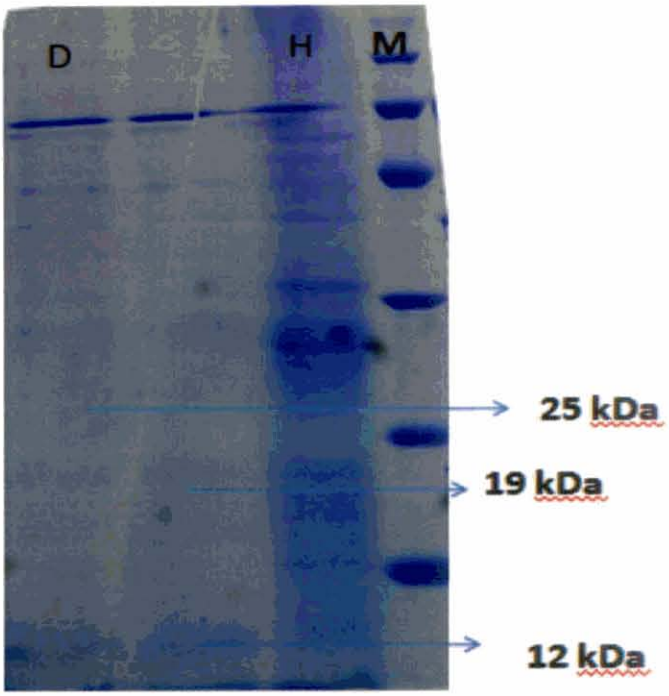
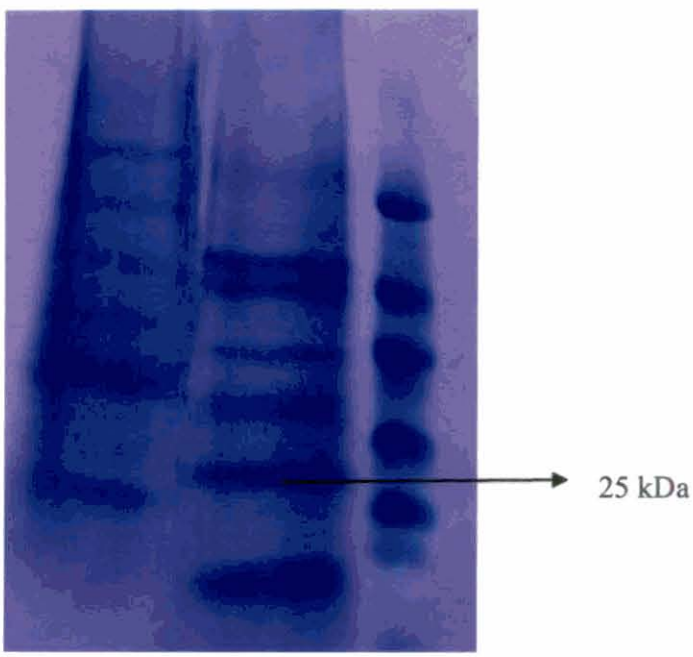


Plate 24. SDS PAGE analysis of healthy and BSV infected samples



H - HEALTHY

M - MARKER

D - DISEASED

Pate 25. SDS PAGE analysis of healthy and CMV infected

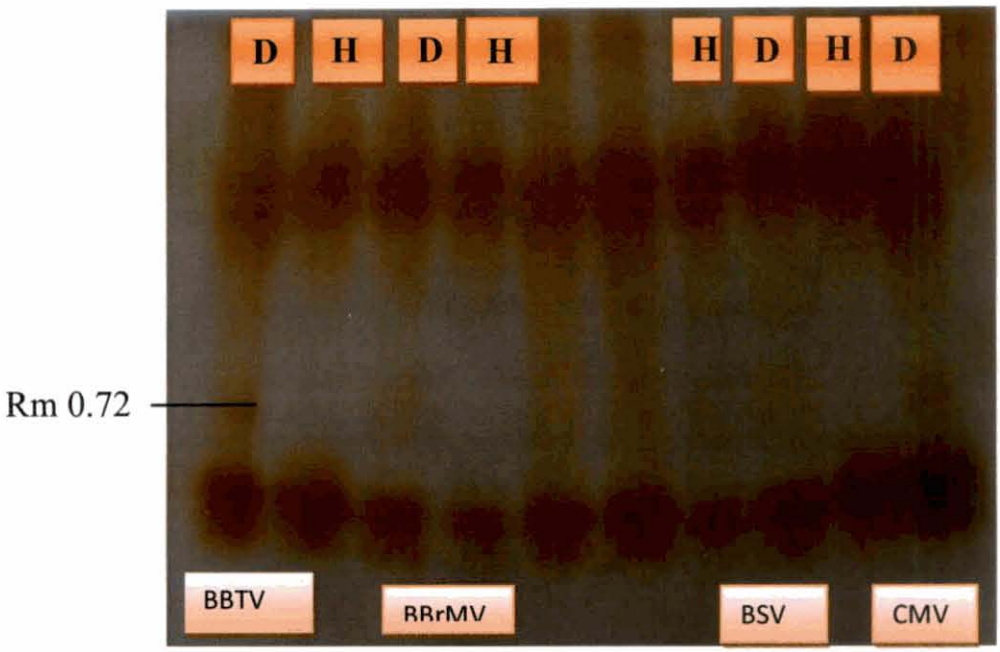


Plate 26. Isozyme analysis of infected and healthy sample

4.3.3 ISOZYME ANALYSIS

Native polyacrylamide gel electrophoresis was carried out for isozyme analysis of peroxidase to find out the variation in isozyme content of healthy and diseased plants. The relative mobility of each band was calculated and Rm was represented as Zymogram. The peroxidase activity in all the four virus infected samples showed the presence of definite single band with Rm value of 0.72. The PO activity in both infected and healthy sample produced two bands with Rm value of 0.86 and 0.33 (Plate 26).

4.3 IMMUNOLOGICAL AND MOLECULAR DIAGNOSIS

4.3.1 IMMUNOLOGICL DETECTION

4.3.1.1 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

DAC-ELISA was done for different infected leaves samples collected from field in different places. Polyclonal antibody (Agdia Pvt. Ltd) was used for detecting the presence of BBTV, BBrMV, BSV and CMV infected samples. Monoclonal antibody was also used for the detection of BSV. The absorbance was measured at 405 nm in an ELISA reader (BIORAD Micro plate Reader). The result of the experiment (Table 8) revealed that out of the thirteen samples analysed, nine samples showed mixed infection. Eight samples gave positive reaction towards BBrMV and CMV antiserum, where one sample gave positive reaction towards BBTV, BSV and CMV antiserum. Two samples reacted only to BBrMV antiserum. Similarly one sample reacted only to BSV antiserum.

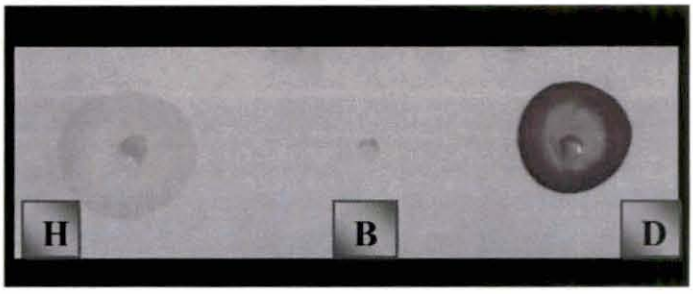
4.3.1.2 DOT IMMUNOBINDING ASSAY (DIBA)

DIBA was conducted to detect the presence of BBrMV, BSV, and CMV infecting banana. Polyclonal antibody for BBrMV, CMV, BSV and monoclonal antibody for BSV were used for the study. The result of the experiment was

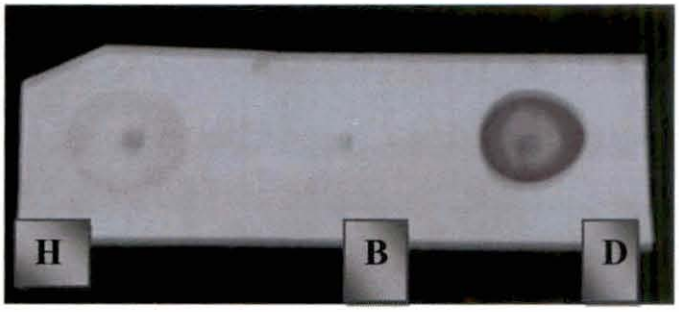
Table 8. Reaction of banana samples infected by virus in DAC - ELISA

Sl. No.	Treatments (different symptoms taken)	Absorbance at 405nm (mean of three replication)					
		Antiserum used					Remarks
		BBTV	BBrMV	BSV (mono)	BSV (Poly)	CMV	
	Healthy	0.258	0.003	0.003	-	0.001	
1.	Kazhakkootam 1	0	0.2406	0.003	-	0.004	+ ve to BBrMV
2	Kazhakkootam 2	0	0.086	0.002	-	0.002	+ ve to BBrMV and CMV
3	Kazhakkootam 3	0	0.035	0.008	-	0.01	+ ve to BBrMV and CMV
4	Kazhakkootam 4	0	0.078	0.005	-	0.024	+ ve to BBrMV and CMV
5	Kazhakkootam 5	0	0.167	0.004	-	0.006	+ ve to BBrMV and CMV
6	Kazhakkootam 6	0	0.069	0.005	-	0.011	+ ve to BBrMV and CMV
7	Kazhakkootam 7	0	0.009	0.005	-	0.0103	+ ve to BBrMV and CMV
8	Kazhakkootam 8	0	0.235	0.004	-	0.002	+ ve to BBrMV
	Healthy	0.253	0.005	-	0.07	0.007	
9	Idukki	0.351	0.211	-	0.071	0.072	+ ve to BBrMV and CMV
10	Vellayani 1	0.329	0.042	-	0.13	0.05	+ ve to BBrMV and CMV
11	Vellayani 2	0.233	0.006	-	0.149	0.011	+ve to BSV
12	Vellayani 3	0.5126	0.011	-	0.175	0.03	+ve to BBTV, BSV, CMV
13	Vellayani 4	0	0	-	0.172	0.004	+ve to BSV

Plate 27 . Dot Immuno Binding Assay (DIBA)



Reaction of BBrMV in banana leaves



Reaction of CMV in banana leaves

Reaction of BSV in banana leaves

D – Diseased

H – Healthy

B – Buffer

analysed by visual observation by comparing development of colour. Reaction of infected samples with polyclonal antibody showed purple coloured spots in the nitro cellulose membrane (NCM) (Plate 27), where the healthy samples and buffer check gave colourless reaction. Monoclonal antibody did not produce colour reaction in the NCM.

4.3.2 MOLECULAR DIAGNOSIS

Molecular diagnosis using PCR was performed for the detection of BBTV and BSV genome, where RT-PCR was performed for the detection of CMV and BBrMV genome. RNA was isolated by using the protocol described by Salzman et al (1999) (Plate 28).

Two gene specific primers were used for detection of BBTV genome. One primer pair was specific for coat protein gene of BBTV where the other was specific for nuclear shuttle protein gene of BBTV. Amplification of coat protein gene and nuclear shuttle protein gene was observed. PCR product of size about 530 bp was obtained with coat protein specific primers (Plate 29). An amplicon of size about 237 bp was observed for NSP gene specific primer (Plate 30).

Two primer pairs specific for coat protein gene were used for the detection of BSV. Amplification using the primers BSV4673 and BSV 5317 yielded the PCR product with amplicon size of 644 bp (Plate 31). Amplification using the primers BSV F and BSV R produced an amplicon of size 730 bp (Plate 32).

Primer pair specific for coat protein gene was used for the detection of CMV infecting banana. Amplification was obtained for the coat protein gene specific primer. The annealing temperature 57.8 °C resulted in maximum amplification. PCR product with size of about 657 bp was observed (Plate 33).

Two primers specific for coat protein gene were used for the detection of BBrMV infecting banana. None of the primer gave amplification in the PCR.

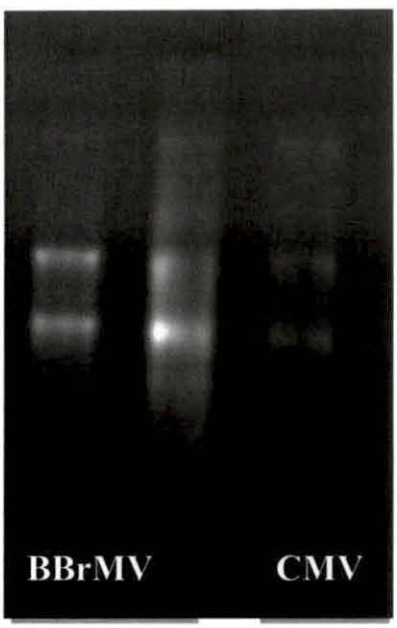


Plate 28. RNA isolated

Plate 29. PCR : Detection of BBTV using CP gene specific primer.

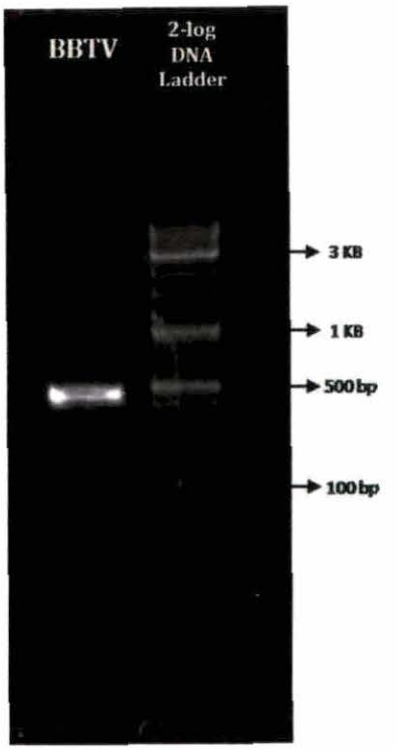


Plate 30. PCR: Detection of BBTV using NSP gene specific primer.

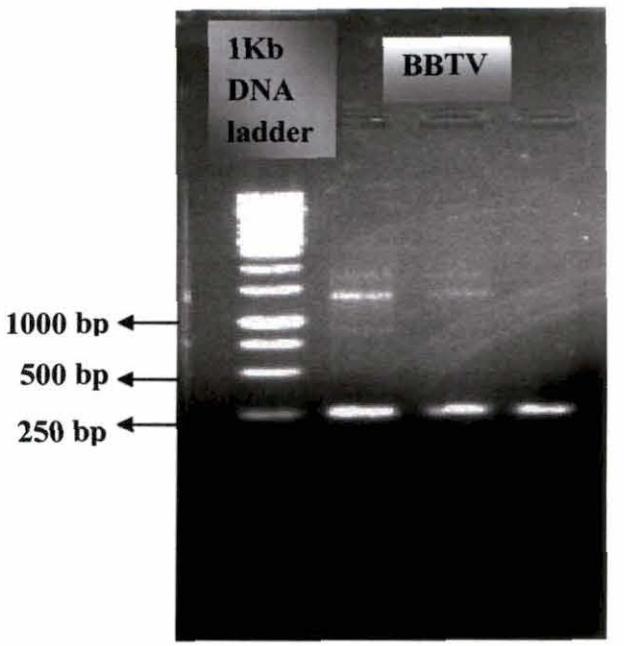


Plate 31. PCR: Detection of BSV using the primers BSV4673 and BSV 5317

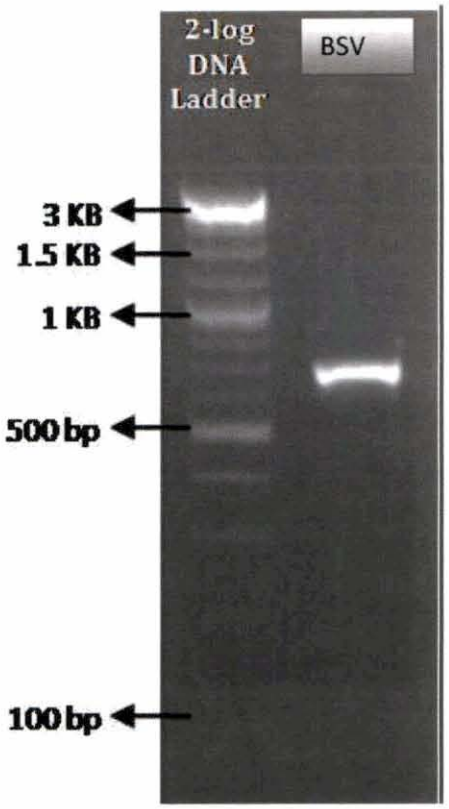


Plate 32. PCR: Detection of BSV using the primers BSVF and BSV R

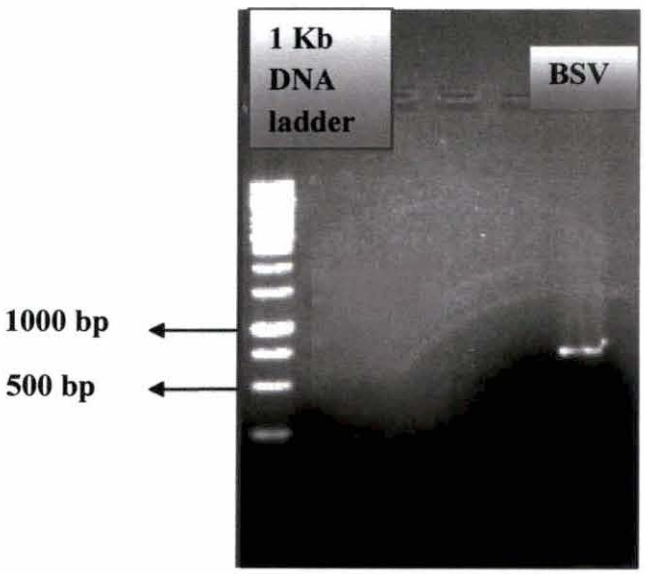
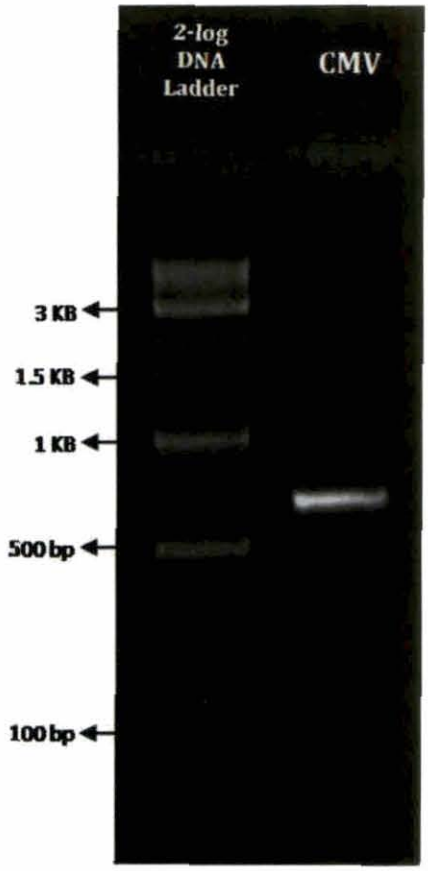


Plate 33. PCR: Detection of CMV using CP gene specific primer.



4.3 Sequencing of the PCR product

The PCR products of BBTV isolate (Appendix X), BSV isolate (Appendix XI) and CMV isolate (Appendix XII) were sequenced. The sequence were analysed using BLAST software (Plate 34, 35, 36). Ten related sequences were selected (Table 9, 10, 11) and cluster dendrogams were constructed.

The result of the cluster dendrogram (Plate 37) revealed that BBTV isolate was mostly related to BBTV coat protein gene of Burundi isolate, Malawi isolate and Rwanda isolate (138 segment). The BBTV isolate was distinctly related to BBTV coat protein of Rwanda isolate (148 segment), Andhaman isolate, Assam isolate, Lucknow isolate.

The result of cluster dendrogram (Plate 38) revealed that BSV isolate was mostly related to Banana streak virus isolate Trichi. BSV isolate was distinctly related distinctly the Banana streak OL virus isolate India (BSOLV-IN1) and Musa balbisiana genomic DNA, complete sequence.

The result of cluster dendrogram (Plate 39) revealed that CMV isolate was mostly related to Cucumber mosaic virus isolate Trichi coat protein gene and Cucumber mosaic virus isolate Calicut coat protein gene. CMV isolate were distinctly related to CMV CP gene of Lucknow isolate and CMV CP gene of Maharashtra isolate.

4.5 ELIMINATION OF VIRUS THROUGH MERISTEM CULTURE

The meristematic region of the virus infected banana suckers were excised and inoculated to MS media with BAP (4 ppm) and NAA (1.5 ppm). The regeneration of plants from meristematic region was difficult because of high phenol content and contamination by endogenous bacteria. The number of regenerated plants from meristem was one. The reduction in meristem size reduced the number of regenerated plants.

BLAST ®

Basic Local Alignment Search Tool

NCBI/ BLAST/ blastn suite/ **Formatting Results - D78CNEH2014**
Formatting options
Download
Blast report description

SR135-BBTV

RID D78CNEH2014 (Expires on 01-14 15:19 pm)

Query ID lcl|60199
Description SR135-BBTV
Molecule type nucleic acid
Query Length 431

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.2.29+

Graphic Summary

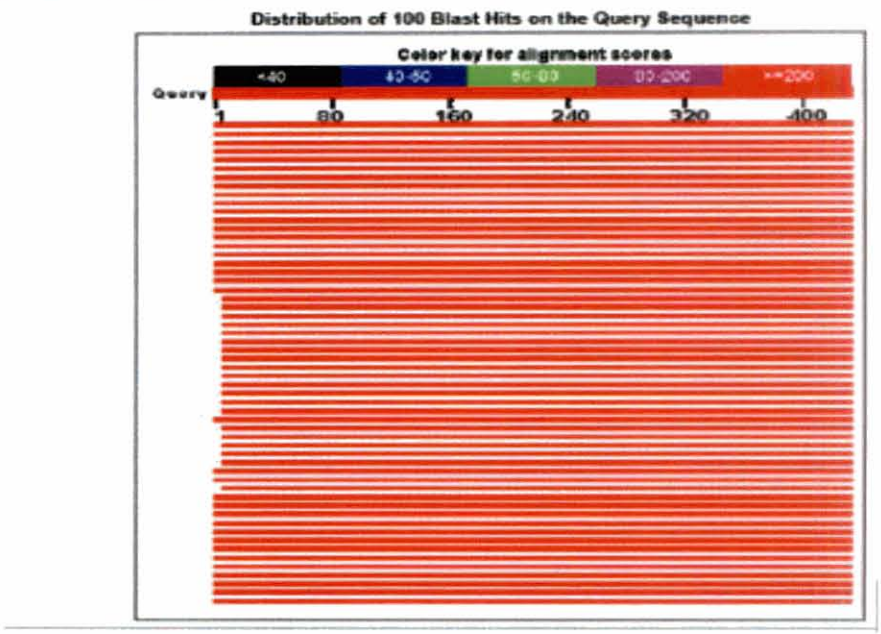


Plate 34. Blast result 0f BBTV isolate

1/13/14 NCBI Blast:SR139

BLAST ®

Basic Local Alignment Search Tool

NCBI BLAST/ blastn suite/ Formatting Results - D79JVS0K014
Formatting options
Download
Blast report description

SR139

RID D79JVS0K014 (Expires on 01-14 15:39 pm)
Query ID lcl|46239
Description SR139
Molecule type nucleic acid
Query Length 603

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.2.29+

Graphic Summary

Distribution of 38 Blast Hits on the Query Sequence

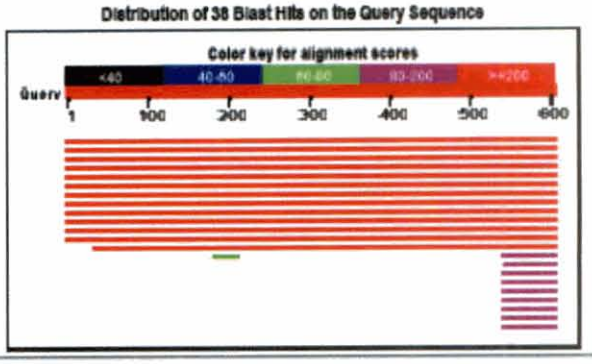


Plate 35. BLAST result of BSV isolate

BLAST ®

Basic Local Alignment Search Tool

NCBI/ BLAST/ blastn suite/ **Formatting Results - DCPYRP0Z014**
Formatting options
Download
Blast report description

SR150-CMV

RID DCPYRP0Z014 (Expires on 01-16 16:58 pm)
Query ID lcl|4231
Description SR150-CMV
Molecule type nucleic acid
Query Length 612

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.2.29+

Graphic Summary

Distribution of 100 Blast Hits on the Query Sequence

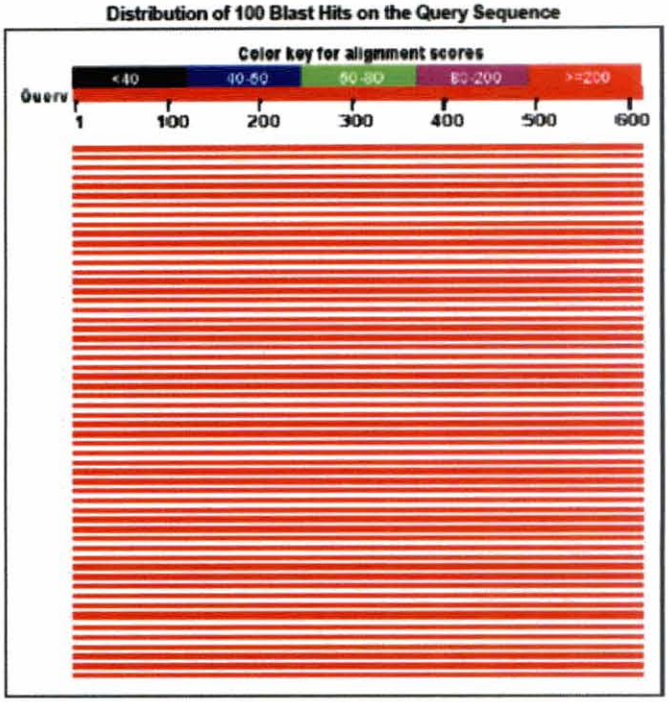


Plate 36. BLAST result of CMV isolate.=

Table 9. Description of selected sequences for dendrogram construction of BBTV isolate.

Sl. No:	Accession No:	Description	Query cover	E Value
1	JQ820455.1	Banana bunchy top virus isolate Malawi 73 segment DNA-S	100%	0.0
2	JQ820461.1	Banana bunchy top virus isolate Rwanda 138 segment DNA-S	100%	0.0
3	JQ820467.1	Banana bunchy top virus isolate Rwanda 142 segment DNA-S	100%	0.0
4	GU125410.1	Banana bunchy top virus isolate Andhaman coat protein gene	100%	0.0
5	EU190965.1	Banana bunchy top virus isolate Assam coat protein (cp) gene	100%	0.0
6	FJ168538.1	Banana bunchy top virus isolate BBTV-Hajipur coat protein gene	100%	0.0
7	EF687856.1	Banana bunchy top virus isolate Lucknow segment DNA 3	100%	0.0
8	DQ515970.2	Banana bunchy top virus coat protein (AV1) gene	100%	0.0
9	AY534140.1	Banana bunchy top virus coat protein (cp) gene	100%	0.0
10	AF148943.1	Banana bunchy top virus DNA-3 isolate Burundi coat protein gene	100%	0.0

Table 10. Description of selected sequence for dendogam construction of BSV isolate.

Sl No:	Accession No:	Description	Query cover	E value
1	DQ859899.1	Banana streak virus isolate TRY, complete genome.	100%	0.0
2	AF106946.1	Musa ABB Group clone Musa6 banana streak virus sequence	100%	0.0
3	GQ374511.1	Musa acuminata AAA Group cultivar baxijiao banana streak virus genomic sequence	100%	0.0
4	JQ409540.1	Banana streak OL virus isolate BSOLV-IN2, complete genome	100%	0.0
5	GQ374512.1	Musa acuminata cultivar dijiao banana streak virus genomic sequence >gb|GQ374515.1| Musa ABB Group cultivar dajiao banana streak virus genomic sequence	100%	0.0
6	HE983609.1	Musa balbisiana cv. Pisang Klutuk Wulung BAC MBP-17D14c, complete sequence	100%	0.0
7	GQ374513.1	Musa ABB Group cultivar fenjiao banana streak virus genomic sequence	100%	0.0
8	JQ409539.1	Banana streak OL virus isolate BSOLV-IN1, complete genome	100%	0.0
9	FJ460020.1	Banana streak virus isolate BSV-ZS polyprotein gene, partial cds	100%	0.0
10	AP009334.1	Musa balbisiana genomic DNA, BAC clone:MBP_31O07, complete sequence	100%	0.0

Table 11. Description of selected isolate for the construction o0f CMV isolate.

Sl. No:	Accession No:	Description	Query cover	E value
1	EU531468.1	Cucumber mosaic virus isolate TRY coat protein gene, complete cds	100%	0.0
2	EF583882.1	Cucumber mosaic virus isolate Calicut coat protein gene, complete cds	100%	0.0
3	EU310928.1	Cucumber mosaic virus isolate CIMAP-India C18 coat protein gene, complete cds	100%	0.0
4	DQ914877.1	Cucumber mosaic virus from Rauvolfia serpentina coat protein gene, complete cds	100%	0.0
5	FN552542.1	Cucumber mosaic virus CP gene for coat protein, genomic RNA, isolate A168	100%	0.0
6	EF593026.1	Cucumber mosaic virus isolate Jatropha segment RNA3, complete sequence	100%	0.0
7	DQ640743.1	Cucumber mosaic virus strain CMV Maharastra coat protein (CP) gene, complete cds	100%	0.0
8	DQ910858.1	Cucumber mosaic virus isolate Lucknow coat protein gene, complete cds	100%	0.0
9	EF153737.1	Cucumber mosaic virus isolate J&K coat protein gene, complete cds	100%	0.0

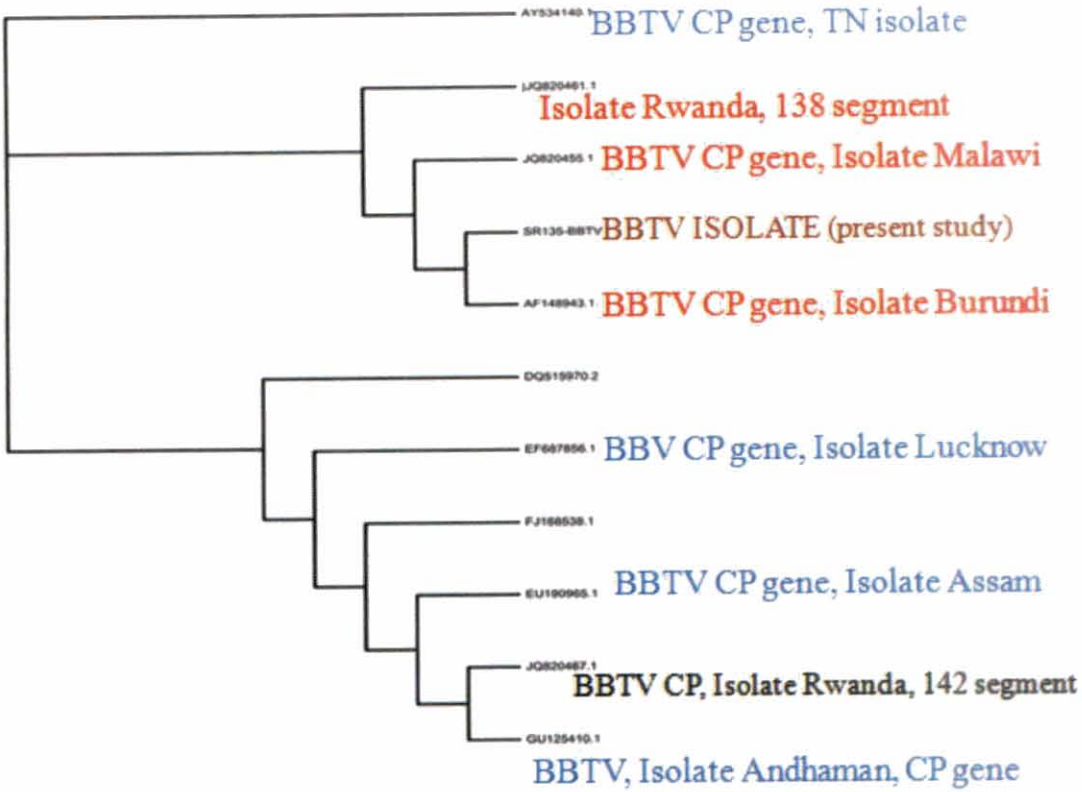


Plate 37. Cluster dendrogram showing the relationship of BBTV isolate with other related sequences.

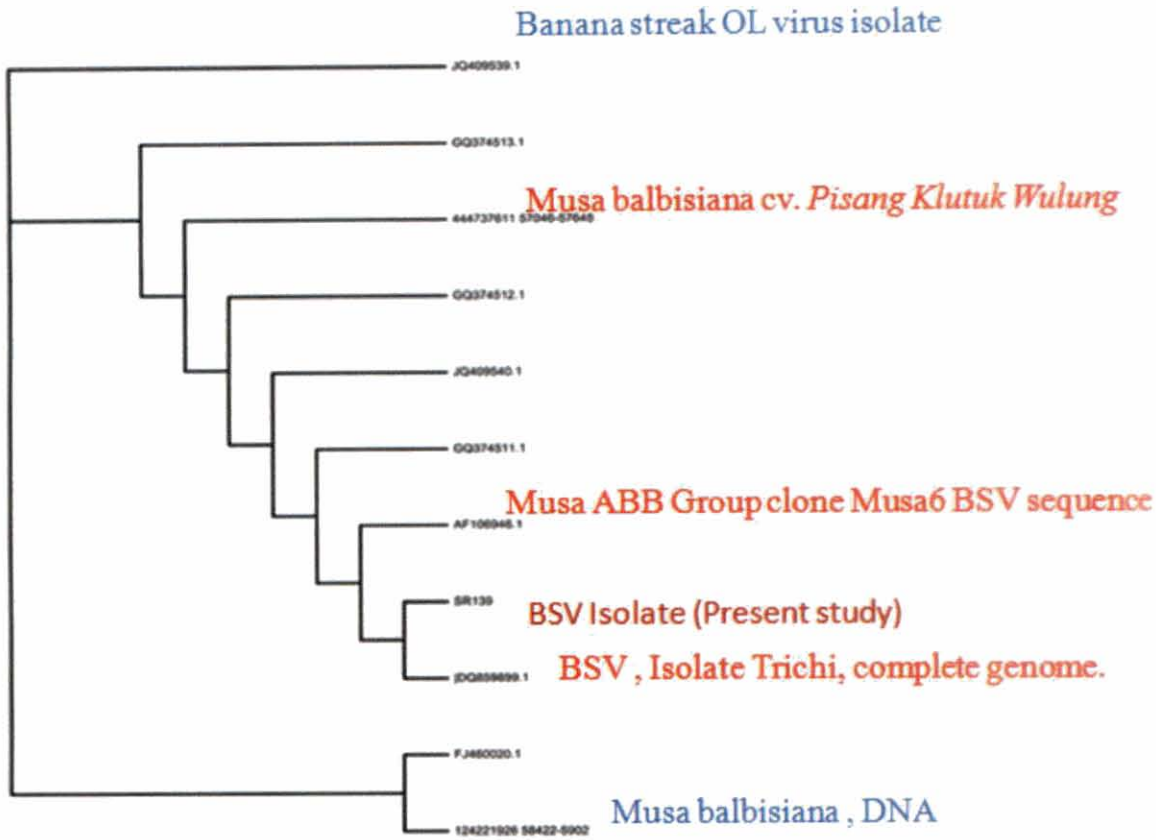


Plate 38. Cluster dendrogram showing the relationship of BSV isolate with other related sequences.

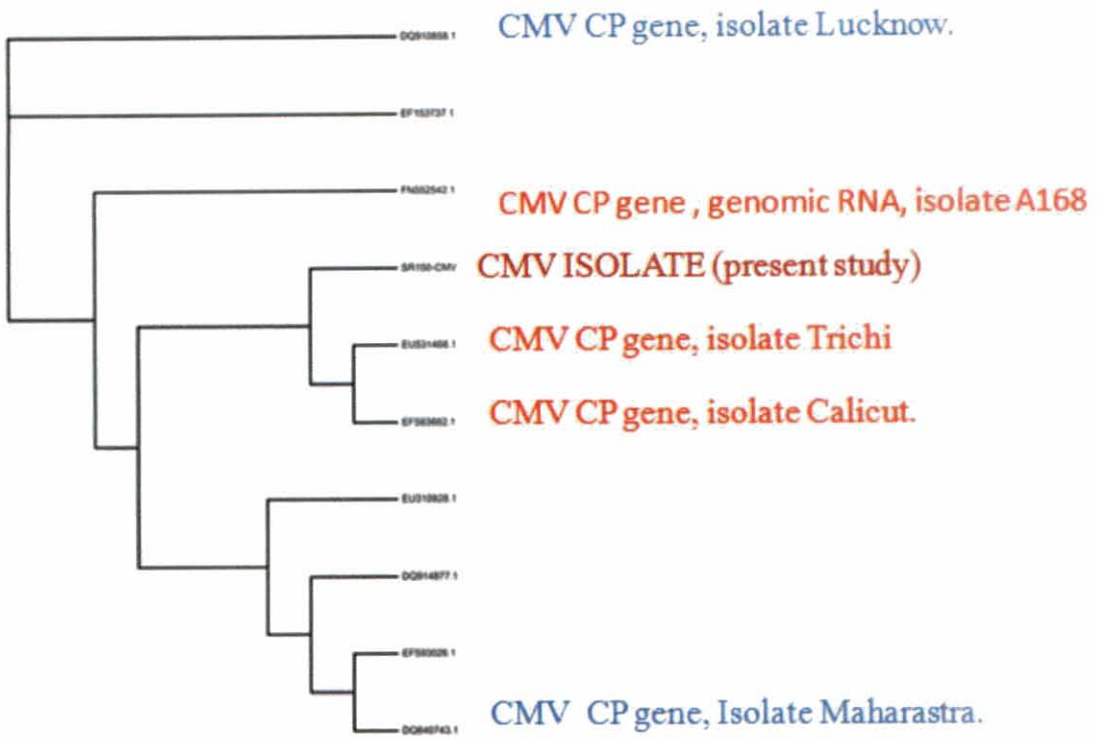


Plate 39. Cluster dendrogram showing the relationship of CMV isolate with other related sequences.

The regenerated plants were subjected to DAC – ELISA and PCR. PCR was done for the detection of BBTV and resulted in no amplification. The plantlets were subjected to DAC – ELISA for the detection of BBrMV, BSV and CMV using the antiserum Poty, ScBV and CMV respectively. The absorbance reading in healthy and tissue culture plants for the antiserum Poty and CMV were almost equal. The absorbance reading in tissue culture for the antiserum ScBV was three times more than that of healthy. So the meristem culture eliminated the virus BBTV, BBrMV, CMV but not the BSV.

Table 12. Reaction of plantlets developed from meristem of infected plants in DAC – ELISA.

Antiserum	Absorbance @ 405 nm	
	Healthy	Tissue culture plants
CMV	0.001	0.002
Poty	0.004	0.003
ScBV	0007	0.027

Discussion

5. DISCUSSION

5.1. SYMPTOMATOLOGY

The characteristic symptoms of the banana bunchy top disease (BBTD) under study were small and brittle leaves with thick veins, bunching of the leaves at the top of the pseudostem. Plants with later infection produced bunch with reduced size. The suckers from infected mother plant were short with bunched leaves and they did not produce fruits.

These symptoms were found to be similar to those caused by BBTV reported earlier. Nelson (2004) described the symptoms of BBTD in suckers as stunting of plants with leaves bunched at the top of the pseudostem. The infected leaves were stiff, erect, shorter and narrower than healthy leaves. Suckers with this symptom did not bear fruits. Plants with later infection produced fruits with distorted hands and fingers.

The characteristic symptom of the Banana Bract Mosaic Disease under study were the appearance of reddish spindle shaped lesion in the pseudostem, bract, leaf petiole and bunch stalk. The infected leaves had chlorotic spindle shaped lesion in the leaf lamina. The phyllotaxy of infected plants were look like that of travellers palm. The infected plant gave low yield than healthy plants.

These symptoms were found to be similar to those caused by BBrMV reported earlier. Rodoni *et al.* (1997) described symptoms due to BBrMV in Coimbatore region as distinctive streaks in the bract of banana inflorescence and mosaic on leaf petiole. Thomas and Magnaye (1996) described symptoms of BBrMV as reddish brown mosaic pattern on the bract, petiole, pseudostem and congested leaf arrangement. Infected leaf lamina showed spindle shaped chlorotic streaks. Magnaye and Espino (1990) described symptom of BBrMV as spindle shaped streaks in the pseudostem.

The characteristic symptom of the banana streak disease under study was chlorotic streaks parallel to the veins in the leaf lamina. Later the chlorotic streaks became necrotic. These symptoms were found to be similar to those caused by

BSV reported earlier. Lockhart (1986) described symptoms of BSV as chlorotic leaf steaks, which became necrotic. Jones and Lockhart (1993) reported symptoms of BSV as yellow streaks on the leaves. Cherian *et al.* (2003) observed chlorotic streak and black streak as symptom of BSV in two accession BRS1 (AAB) and Mysore poovan (AAB). Dahal *et al.* (2000) described the symptoms of BSV infecting different *Musa* genotypes in Nigeria as discrete yellow streaks on the leaves. Ndowora *et al.* (2000) described symptoms due to BSV infection as yellow and necrotic leaf streaks.

The characteristic symptoms due to CMV infection were chlorosis on the leaf lamina as initial symptom and necrosis of the spindle leaf. Later the chlorosis developed in to mosaic pattern on entire leaf lamina. These symptoms were found to be similar to those caused by CMV reported earlier. Lockhart (2002) described symptoms due to heart rot stains of CMV in banana as chlorosis, cigar leaf necrosis and plant death. Vishnoi *et al.* (2013) described the symptoms due to CMV as severe mosaic and leaf deformation.

5.2 HOST PATHOGEN INTERACTION

Physiological changes in banana cv. Nendran due to viral infection were studied with respect to the content of total carbohydrate, chlorophyll, total phenol, protein by comparing with healthy plant.

There was significant difference in total carbohydrate content between healthy and infected plant. The total carbohydrate content of BBTV, BBrMV, BSV infected plants were less than that of healthy, where CMV infected plants showed more carbohydrate content. Alagiamanavalan *et al.* (1973) reported that there was an increase in reducing and non – reducing sugars and pectin content in BBTV affected fruits. Increased sugar content in okra plant inoculated with *Yellow vein mosaic virus* was reported by Bhagat and Yadav (1997). They opined that accumulation of sugar was due to the disruption of normal phloem transport. Total carbohydrate content of BBrMV infected leaves were less than healthy ones at three month after planting (MAP), six MAP, and flag leaf stage (Dhanya,

2004). Smitha (2001) reported higher carbohydrate content in the leaves of healthy banana compared to that of BBrMV infected plants. Mali *et al.* (2000) reported that infection of yellow mosaic virus in moth bean resulted in reduction of total soluble carbohydrate. The reduction in the level of carbohydrate was due to the breakdown of carbohydrate which is accelerated during respiration in virus infected plants as suggested by Narayanaswamy and Ramakrishnan (1966). Earlier reports explain that there was increase and decrease of carbohydrate content in infected plant. Increased carbohydrate content may be due to the disruption of phloem transport. Decreased carbohydrate content may be due to the breakdown of carbohydrate. In the present investigation the enhanced carbohydrate content in CMV infected plant may be due to the interaction of this virus with the host.

The total chlorophyll, chlorophyll a and chlorophyll b content were significantly less in BBTV, BBrMV, BSV and CMV infected banana plants when compared to healthy plants. Maximum reduction in chlorophyll a (79%) and total chlorophyll (71%) content was showed by CMV infected plants. Maximum reduction in chlorophyll b was showed by BSV and CMV infected plants ie, 74%. Hook *et al.* (2007) reported that chlorophyll a and chlorophyll b content in banana leaves infected with BBTV was significantly reduced compared to healthy plant. Yasmin *et al.* (2001) investigated the effect of BBTV on leaf chlorophyll content of banana and found that due to the infection, there was severe reduction in total chlorophyll, chlorophyll a, chlorophyll b over healthy control. Total chlorophyll content was found to be less at all stages of BBrMV infected banana leaves than healthy ones (Dhanya, 2004). Devi and Radha (2012) observed reduction in chlorophyll a, chlorophyll b and total chlorophyll in cucurbits plants infected with CMV. Virus infection affects photosynthesis in a complex manner, depending on the particular host-virus combination (Almasi *et al.*, 2001). The reduction in chlorophyll is due to enhanced chlorophyllase activity or could be due to the inhibition of chlorophyll synthesis (Palaniswamy *et al.*, 2011). The above finding supports the present study that viral infection resulted in decreased

chlorophyll content. This may be due to the enhanced chlorophyllase activity or due to the inhibition of chlorophyll synthesis.

There was significant difference in phenol content between healthy and infected banana plants. Infected plants showed more phenol content than healthy plants. Total phenol was reported to be high in virus infected leaves of many plants (Srivastava and Tiwari, 1998). Smitha (2001) reported that the BBrMV infected plants had more phenol content than healthy ones. Dhanya (2004) observed increased phenol content in BBrMV infected banana leaves than healthy ones in all growth stages. The total phenols in cucumber plant were significantly increased as a consequence of *Cucumber mosaic virus* (CMV) infection (Devi and Radha, 2012). Jeeva (2001) reported that sweet potato feathery mottle virus infection on sweet potato led to an increase in activity of hexose monophosphate (HMP) shunt pathway. HMP pathway is required for the synthesis of intermediaries needed for phenol synthesis. These earlier studies support the present study that viral infection resulted in increased phenol content. This may be due to the increase action of HMP pathway due to viral infection.

Changes in protein content due to viral infection were studied in virus infected banana plants. The protein content was higher in infected sample compared to healthy ones. Estelitta (1998) reported higher percentage of protein in banana bunchy top affected plants than healthy plants. BBrMV infected leaves were found to have more protein content than healthy ones in all growth stages. Both healthy and infected samples showed decreasing trend in protein content in all growth stages till bunch formation (Dhanya, 2004). CMV and pepper yellow mottle virus (PYMoV) infected leaves of pepper showed increased protein content than healthy ones in all growth stages. The highest protein content was recorded at six month after grafting (MAG) (Ayisha, 2010). Protein content was found to be higher in cucumber plants infected with CMV. Healthy plants the protein content was 34 µg per gram while in treated plants it varied from 36 to59 µg per gram (Devi and Radha, 2012). The increase in total protein content in the virus inoculated plants at different stages was due to increase in viral proteins and non-viral induced proteins as reported by Kovalenko and Shepelevitch (2003). All

these reports are in conformity with those obtained in the present investigation. The increase in protein content in BBTV, BBrMV , BSV, CMV infected plant may be due to the viral proteins or due to the virus induced proteins.

Changes in activities of defence related enzymes like peroxidase, polyphenol oxidase, phenyl alanine ammonium lyase in virus infected banana were studied. In the present study, the activity of peroxidase, polyphenol oxidase and phenyl alanine ammonia lyase activity was more in BBTV, BBrMV, BSV and CMV infected plants than that of healthy plants. Highest peroxidase, polyphenol oxidase, phenyl alanine ammonialyase activity was showed by BBTV, BBrMV and CMV infected plants respectively. Smitha (2001) found enhanced activity of PO, PPO and PAL in BBrMV infected banana plants compared to healthy ones. Dhanya (2004) observed increased peroxidase (PO), poly phenol oxidase (PPO) activities in BBrMV infected banana samples in all growth stages. Gomathi *et al.* (1993) observed enhanced activity of PO, PPO, PAL in BSV and CMV infected banana plants. Defence related enzymes were reported to act as an important factor in the induction of resistance (Dasgupta, 1988). Ahmed *et al.* (1992) suggested that higher amount of phenols, oxidation products like quinones and increased activity of PO and PPO might be responsible for reduced virus multiplication in okra infected with *Bhindi Yellow vein mosaic virus*. These reports are in agrement with the present study. The increased activity of defence related enzymes in the present study may be due to the resistant reaction of the infected plant.

Viral infection leads to the appearance of specific soluble proteins. Protein profile of healthy and infected banana samples due to BBTV, BBrMV, BSV and CMV were analysed using SDS-PAGE. The virus infected sample showed extra band in their protein profile. The protein profile of BBTV infected sample showed one extra band with molecular weight of approximately 20 kDa. The protein profile of BBrMV infected sample showed two major protein band with molecular weight of 38 kDa and 29 kDa and one minor protein band with molecular weight of 22 kDa. The protein profile of BSV infected sample showed three extra protein bands. The molecular weight of these additional proteins was 25 kDa, 19 kDa, and

12 kDa. The protein profile of CMV infected sample showed one extra band with molecular weight of 25 kDa.

Harding *et al.* (1991) analysed the size of coat protein of BBTV by doing SDS PAGE and silverstaining. The results revealed a protein with a molecular mass of 20 kDa. Abdelkader *et al.*, (2004) performed SDS PAGE for determining size of purified coat protein of BBTV particles and found that the size is 21kDa. These earlier reports support the present study. In the present study, one extra protein band were found in BBTV infected sample with molecular weight of 20 kDa. This protein may be due to viral coat protein.

The coat protein of the BBrMV resolved as one major polypeptide with an estimated molecular weight (M_r) of 38 kDa and three minor polypeptides with molecular weight of 63, 53 and 22 kDa. in a 12 % SDS-PAGE (Kiranmai *et al.*, 2005). This report supports the present study. In the present study also got extra protein band with molecular weight of 38 kDa and 22 kDa. This extra protein band may be due o the viral coat protein.

Khan *et al.* (2011) performed SDS-PAGE to determine the size of coat protein (cp) in *Cucumber mosaic virus* (CMV) and the result revealed that the size is 25kDa. This report supports the present study. In the present study also got extra protein band with molecular weight of 25 kDa, this may be due to viral coat protein.

Electrophoretic analysis of isozyme was done to study the enzyme alteration in healthy and infected banana leaves. In the present study isozyme analysis was done for the study of peroxidase activity. The peroxidase activity in BBTV, BBrMV, BSV and CMV infected samples showed the presence of definite single band with Rm value of 0.72. The PO activity in both infected and healthy sample produced two bands with Rm value of 0.86 and 0.33. Dhanya *et al.* (2006) conducted native polyacrilamide gel electrophoretic analysis of isozyme in BBrMV infected plants as well as healthy plants and found single definite isoforms of polyphenol oxidase (Rm 0.63) in diseased plant. The change in the isozyme spectrum in various host virus combination is mainly due to the host tissue and not by the virus (Solymosy *et al.*, 1967). The earlier report supports the

present study. The increased activity of peroxidase in the infected sample may be due to the changes in host mechanism as a result of host pathogen interactions.

5.3 IMMUNOLOGICAL AND MOLECULAR DIAGNOSIS

Serological techniques are useful for the identification, quantitative assay and routine diagnosis of plant viruses. In the present study DAC – ELISA and DIBA were found to sensitive diagnostic techniques for the detection of BBTV, BSV, BBMV and CMV infecting banana. In the present study DAC – ELISA was performed for the detection of virus infected samples collected from different location using polyclonal and monoclonal antiserum. Out of thirteen infected samples analysed, nine samples showed mixed infection with more than one virus. Prakash *et al.* (2010) reported that ELISA was good enough to discriminate BBTV, BBrMV, BSV, CMV infected suckers and micro propagated samples from healthy ones. Mariappan and Mathikumar (1992) reported that ELISA was the most sensitive method for detecting BBTV at very low virus concentration. DAC-ELISA was found to be more economical serological method in detecting BBrMV (Dhanya *et al.*, 2007). Ndowora *et al.*, (2000) diagnosed BSV by DAS-ELISA and triple antibody sandwich enzyme immunoassay (TAS-EIA) using polyclonal antibody raised against SCBV and BSV isolate from 'Mysore' variety. Dheepa and Paranjothi (2010) serologically diagnosed CMV infecting banana using DAC ELISA. These reports support the present study to suggest that DAC – ELISA is a very effective in detecting BBTV, BBrMV, BSV and CMV infecting banana.

DIBA was used for the detection of BBrMV, CMV and BSV infecting banana. DIBA successfully detected the presence of BBrMV, BSV and CMV infecting banana. Selvarajan (1997) detected BBrMV in infected samples using DIBA technique. Abdel – Salam *et al.*, (2005) detected BSV infected banana samples using dot and tissue blot immuno assays with antiserum raised against Egyptian isolate of BSV. Rajasulochana *et al.* (2008) reported that Dot-blot-ELISA was relatively more sensitive for the detection of BSV and CMV in banana than ELISA. These reports support the present study that DIBA is an

equally effective technique in detecting BSV, CMV and BBrMV infecting banana.

Detection of two DNA viruses ie, BBTV & BSV infecting banana were performed using PCR. Detection of two RNA viruses ie, BBrMV & CMV infecting banana were performed using Reverse transcription –PCR (RT – PCR). In the present study the presence of BBTV, BSV, and CMV was confirmed using PCR technique. The coat protein (CP) gene specific primer and nuclear shuttle protein (NSP) gene specific primer were used for the detection of BBTV. The CP gene specific primer resulted in amplified product with size of ~ 530 bp where as NSP gene specific primer resulted in amplified product with a size of 237 bp. Two CP gene specific primers were used for the detection of BSV. The primer BSV 4673 & BSV 5317 (Manoranjith *et al.*, 2012) resulted in PCR product with size of 644 bp. where the primers BSV F & BSV R (Cherian *et al.*, 2003) resulted in amplified product with size of 730 bp. CP gene specific primer used for the detection of CMV, resulted in an amplicon size of 687 bp. Two CP gene specific primer were used for the detection of BBrMV, none of the primer could amplify the gene in the present investigation.

Mahadev *et al.* (2013) performed PCR for the detection of BBTV in symptomless plant and tissue culture plants. They used CP gene specific primer and NSP gene specific primer which resulted in an amplified product with an amplicon size of 530 bp and 237 bp respectively. This report is in conformity with the results obtained for the BBTV isolate in the present study.

Manoranjith *et al.*, (2012) performed PCR for the detection of BSV Tamil Nadu isolate using primers designed for the amplification of BSV Nigerian isolate, which resulted in PCR amplified product with a size of 644bp. Cherian *et al.* (2003) amplified the BSV Kerala isolate using primer designed from sequence data of Nigerian isolate with an amplicon of size 730bp. These reports are in conformity with the results obtained in the present study. The present investigation on nucleic acid based detection confirmed that the BSV isolate in the infected sample was closely related to Tamilnadu isolate and Kerala isolate, which inturn related to Nigerian isolate.

Khan *et al.* (2011) performed reverse transcription PCR (RT – PCR) for the detection of CMV in Karnataka (KAR), Utter Pradesh (UP), Maharashtra (MH) using CP gene specific primer which resulted in amplification of 657 bp. These reports are in conformity with the results obtained in the present study. Hence the present investigation on nucleic acid based detection confirmed that the CMV isolate in the present study was closely related to KAR, UP, MH isolate.

Rodoni *et al.*, (1997) amplified BBrMV infecting banana in southern India and found an amplification product of ~604 bp. Dassanayake (2001) amplified Srilankan isolate of BBrMV using CP gene specific primer yielding a product of ~324 bp. In the present study both the primers used, did not amplify the BBrMV isolate. Designing of new primer pairs are is needed for the amplification of BBrMV Vellayani isolate.

5.4 Sequencing

PCR product was analysed using BLAST software and dendrograms were constructed. The result of cluster dendrograms of BBTV isolate revealed that it was related to BBTV coat protein gene of Burundi isolate and distinctively related to Assam, Lucknow isolate of BBTV CP gene. Selvarjan *et al.*, (2011) reported that there was a significantly higher degree of divergence (up to 4%) between amino acid sequences of BBTV CP among Indian isolates. This report support the present study that the differences in aminoacid sequence in BBTV CP gene among the Indian isolate may be the reason for the distinct relationship of BBTV isolate in present study with the other Indian isolate.

The result of cluster dendrograms of BSV isolate revealed that it was related to BSV coat protein gene of Trichi isolate. Cherian *et al.*, (2003) reported that the BSV K3 from banana cv Mysore Poovan was identical to Nigerian isolate of BSV, sharing an identity of 99%, BSV K1 from banana BRS-1 was identical to Nigerian isolate sharing identity of 97%. Manoranjith *et al.*, (2012) successfully amplified BSV Tamil Nadu isolate using primers designed for the amplification of BSV Nigerian isolate. These reports are in conformity with the results obtained in the present study. Hence the BSV isolate used in the present study was related to

BSV isolate in Tamil Nadu, Kerala and Nigerian isolate. The BSV isolate in the present study was also related to *Musa balbisiana* cv. *Pisang Klutuk Wulung.* Harper *et al.* (1999) reported that BSV viral sequence may integrate in to the host nuclear genome. This report is in agreement with the present study, where the BSV isolate may in an integrated form.

The result of cluster dendrograms of CMV isolate revealed that it was related to CMV coat protein gene of Trichi isolate infecting banana and CMV coat protein infecting paprika. Khan *et al.* (2011) sequenced CMV infecting banana from Karnataka, Maharashtra, and Uttar Pradesh in India and found that 93%- 98% sequence similarity at nucleotide level. They also report that these sequences also showed similarity with CMV infecting *Physalis minima.* This report support the present study that the CMV isolated was infecting banana is the same infecting cucurbitaceous crops.

5.5 ELIMINATION OF VIRUS THROUGH MERISTEM CULTURE

Meristem culture was attempted to find out the possibility of eliminating the virus from infected planting material. The regeneration of plants from meristematic region was difficult because of high phenol production and contamination by endogenous bacteria. The plantlets were subjected to PCR and DAC – ELISA. The result indicated the presence of BSV. The viruses CMV, BBrMV, and BBTV could not be detected in the meristem culture. There were reports of enhanced presence of banana streak virus in tissue of culture plants. Harper *et al.* (1999) reported that BSV viral sequence may integrate in to the host nuclear genome. Due to the wide spread of endogenous para retro virus sequence E (PRVs) of BSV in Musa sp. and interspecific hybrids, BSV has become a main constraint to Musa improvement programme, germplasm exchange and tissue culture (Selvarajan *et al.*, 2011). Tissue culture and breeding methods resulted in the expression of integrated BSV genome (Lockhart, 1997). Shirgai *et al.* (2008) eliminated BBTV from infected plant by using meristem culture. El- Dougdoug *et*

al. (2009) observed banana meristem tip of size 0.3 mm is more effective for BBTV, BMV eradication in *invitro*. Virus-free plants of banana cultivars Poyo and Giant Cavendish infected by CMV were produced by meristem tissue culture (Rivas, 1988). Above reports are in conformity with the present study in that meristem culture could eliminate BBTV, BBrMV and CMV except CMV. The virus indexing will be full proof only if both the immunological and molecular detection protocol is done together.

Summary

6. SUMMARY

The present study was conducted for the diagnosis of BBTV, BBrMV, BSV and CMV infecting banana and production of disease free planting materials of banana variety, Nendran. The experiment was conducted in Department of Plant Pathology, College of Agriculture, Vellayani.

Symptomatological studies showed that the characteristics symptoms caused by BBTV were small, brittle leaves with thickened veins which remained bunched at the top of the pseudostem. Plants with early infection did not produce fruits where plants with later infection produce bunch with reduced size, weight and mishapen fingers. The characteristic symptoms caused by BBrMV were reddish spindle shaped lesion in the pseudostem, flag leaf sheath, leaf petiole, and bract. Leaves of infected plants showed characteristic chlorotic spindle shaped lession on the leaf lamina. The infected plant gave low yield than healthy plant. Some infected plants produced travellers palm like appearance. The suckers from infected mother plant also produced characteristic reddish spindle shaped lesion. The characteristic symptoms of BSV were chlorotic streaks in the leaf lamina. Later the chlorotic streaks became necrotic. Some BSV infected plans showed vertical striation on the leaf lamina The characteristic symptom of CMV was mosaic pattern in the leaf lamina. Initially the mosaic symptoms appeared to be localized, later the mosaic symptom spread on entire leaf lamina. The necrosis of the spindle leaf was also observed.

The pathophysiological studies revealed that the virus infected plants showed significant difference in carbohydrate, chlorophyll, phenol and protein content when compared to healthy ones. The total carbohydrate content of BBTV, BBrMV, and BSV infected banana plants were less than that of healthy plant. The total carbohydrate content of CMV infected plants was more than that of healthy. The virus infected samples showed less chlorophyll content than healthy samples. Enhanced level of phenol content was observed in infected samples when compared

to healthy samples. Compared to healthy the infected plants have more protein content. There was a significance difference in peroxidase, poly phenol oxcidase and phenyl alanine ammonia lyase activity between healthy and infected sample. Infected samples showed more peroxidase, polyphenol oxidase and Phenyl alanine ammonia lyase activity.

Protein profile of healthy and infected banana samples due to BBTV, BBrMV, BSV and CMV were analysed using SDS-PAGE. The virus infected sample showed extra band in their protein profile. The protein profile of BBTV infected sample showed one extra band with molecular weight of approximately 20 kDa. The protein profile of BBrMV infected sample showed two major protein band with molecular weight of 38 kDa and 29 kDa and one minor protein band with molecular weight of 22 kDa. The protein profile of BSV infected sample showed three extra protein bands. The molecular weights of these additional proteins were 25 kDa, 19 kDa, and 12 kDa. The protein profile of CMV infected sample showed one extra band with molecular weight of 25 kDa.

Native polyacrylamide gel electrophoresis was carried out for isozyme analysis of peroxidase to find out the variation in isozyme content of healthy and diseased plants. The peroxidase activity in all the four virus infected samples showed the presence of definite single band with Rm value of 0.72.

DAC-ELISA was done for different infected leaves samples collected from field in different places. Polyclonal antibody (Agdia Pvt. Ltd) was used for detecting the presence of BBTV, BBrMV, BSV and CMV infected samples. Monoclonal antibody was also used for the detection of BSV. The result revealed that out of the thirteen samples analysed, nine sample showed mixed infection.

DIBA was conducted to detect the presence of BBrMV, BSV, and CMV infecting banana. Polyclonal antibody for BBrMV, CMV, BSV and monoclonal

antibody for BSV were used for the study. Monoclonal antibody did not give positive reaction, where polyclonal antibody gave positive reaction in infected samples.

Molecular diagnosis using PCR was performed for the detection of BBTV and BSV genome, where RT-PCR was performed for the detection of CMV and BBrMV genome. Two gene specific primers were used for detection of BBTV genome. PCR product of size about 530 bp was obtained with coat protein specific primers. An amplicon of size about 237 bp was observed for NSP gene specific primer. Two primer pairs specific for coat protein gene were used for the detection of BSV. Amplification using the primers BSV4673 and BSV 5317 yielded the PCR product with amplicon size of 644 bp. Amplification using the primers BSV F and BSV R produced an amplicon of size 730 bp. Primer pair specific for coat protein gene was used for the detection of CMV infecting banana. PCR product with size of about 657 bp was observed. Two primers specific for coat protein gene were used for the detection of BBrMV infecting banana. None of the primer gave amplification in the PCR.

Sequencing result revealed that revealed that BBTV isolate was mostly related to BBTV coat protein gene of Burundi isolate, Malawi isolate and Rwanda isolate (138 segment). The BBTV isolate was distinctly related to BBTV coat protein of Rwanda isolate (148 segment), Andhaman isolate, Assam isolate, Lucknow isolate. BSV isolate was mostly related to Banana streak virus isolate Trichi. BSV isolate was distinctly related distinctly the Banana streak OL virus isolate India (BSOLV-IN1) and Musa balbisiana genomic DNA, complete sequence. CMV isolate was mostly related to Cucumber mosaic virus isolate Trichi coat protein gene and Cucumber mosaic virus isolate Calicut coat protein gene. CMV isolate were distinctly related to CMV CP gene of Lucknow isolate and CMV CP gene of Maharashtra isolate.

The meristematic region of the virus infected banana suckers were excised and inoculated to MS media with BAP and NAA. The regeneration of plants from

meristematic region was difficult because of high phenol production and contamination by endogenous bacteria. Meristem culture eliminated BBTV, CMV and BBrMV but not the BSV.

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Appendices

APPENDIX I

0.1 M sodium acetate (pH 4.7)

Stock solutions

A: 0.2 M solution of acetic acid (11.55 ml in 1000 ml)

B: 0.2 M solution of sodium acetate (16.4 g of $C_2H_3O_2$ Na or 27.2 g of $C_2H_3O_2$ Na $3H_2O$ in 1000 ml).

22.7 ml of A is mixed with 27 ml of B, diluted to a total of 100 ml.

APPENDIX II

Preparation of stock dye solution for estimation of protein

100 mg of coomassie brilliant blue G-250 was dissolved in 50 ml of 95 per cent ethanol and 100ml of concentrated orthophosphoric acid was added. The volume was made up to 200 ml with water and kept at 4^0C. The working dye was prepared just before use by diluting stock solution to five times with water.

APPENDIX III

0.1 M Sodium phosphate buffer (pH 6.5)

Stock solutions

A. 0.2 M Solution of monobasic sodium phosphate (27.8 g in 1000 ml)
B. 02 M solution of di basic sodium phosphate (53.65 g NA2 HPO4. 7H2O in 1000ml)

68.5 ml of A mixed with 31.5 ml of B diluted to a total of 200 ml.

APPENDIX IV

0.1 M Borate Buffer (pH 8.8)

A: 0.2 M solution boric acid (12.4 g in 1000 ml)

B: 0.05 M solution of borax (19.05 g in 1000 ml)

50 ml of A is mixed with 30 ml of B, diluted to a total of 200 ml.

APPENDIX V

Protein denaturing solution

10 M urea - 80 ml

1M NaH2PO42H2O (pH 8) - 5ml

5M sodium chloride - 2 ml

1M Tris (pH 8) - 1ml

Make up volume to 100ml by adding 12mlof distilled water.

APPENDIX VI

Stock solutions for isozyme analysis

1. Tris-glycine electrode buffer stock solution. pH 8.3

   Tris - 6.0 g

   Glycine - 28.8 g

   Distilled water - 1000 ml

2. Electrode buffer

   Dilute the Tris- glycine electrodebuffer stock solution with distilled water in a 1: 9 ratio.

3. Tris- chloride buffer stock solution, pH 8.9

   HCl, 1N - 48.00 ml

   Tris - 36.6 g

   TEMED - 0.23 ml

   Distilled water -100.00 ml

4. Tris- chloride buffer stock, pH 6.7

   HCl, 1N - 48.00 ml

   Tris - 5.98 g

   TEMED - 0.46 ml

   Distilled water - 100.00 ml

5. Resolving gel acrylamide stock solution

   Acrylamide – 28.00 g

Bis-acrylamide – 0.74 g

Double distilled water – 100.0 ml

Store in dark bottle at 4^0C for upto 2 weeks.

6. Ammonium persulphate solution

Ammonium persulphate - 0.1 g

Dissolve in 1 ml distilled water. Prepared freshly before use

7. Bromophenol blue solution

Bromophenol - 25 mg

Make upto to 10 ml with Tris- chloride buffer solution, pH 6.7

8. Resolving gel solution (for one 1.5 mm gel. 7.5 %)

Tris- chloride buffer - 5 ml

Solution, pH 8.9

Resolving gel acrylamide solution - 10 ml

Distilled water - 25 ml

Ammonium persulphate solution - 300 µl

9. Stacking gel solution (for one 1.5 mm gel, 4%)

Tris- chloride buffer stock - 2.5 ml

Solution, pH 6.7

Resolving gel acrylamide solution - 10 ml

Distilled water - 25 ml

Ammonium persulphate solution - 300 µl

APPENDIX VII

Buffers for ELISA

1 Phosphate buffer saline (1 x PBS) pH 7.4

NaCl	-	8 g
$Na_2HPO_4\ 2H_2O$	-	1.44 g
KH_2PO_4	-	0.2g
KCl	-	0.2g
Distilled water to make	-	1 Litre

2 Wash Buffer (PBS-T) - Add 0.5ml/L of Tween-20 to PBS

3 Coating buffer (Carbonate buffer pH 9.6)

Na_2CO_3	-	1.59g
$NaHCO_3$	-	2.93g
Distilled water to make	-	1 Litre

4 Enzyme conjugate diluent buffer (PBS-TPO)

Add 20 g PVP and 2 g ovalbumin to 1 l PBS-T

5 Antibody diluent buffer – Same as PBS-TPO

6 Substrate buffer (pH9.8)

Diethanol amine	-	97 ml
Distilled water	-	800 ml.

APPENDIX VIII

DOT – IMMUNOBINDING ASSAY (DIBA)

Chemicals and solutions

1. Stock buffer (Tris-buffer saline, TBS, pH 7.5)

   0.02 M Tris - 48 g

   0.5 M NaCl - 58.48 g

   Adjust the pH to 7.5 with 1 N HCl and make upto 2 litre. This is used as Wash solution.

2. Antigen extraction buffer (TBS – 50 mM DIECA)

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

3. Blocking solution (TBS-SDM)

   Add 5.0 g spray dried milk (SDM) to 100 ml TBS

4 Antibody and enzyme-conjugate diluent/buffer

   Same as TBS-SDM

5 Substrate buffer (pH 9.5)

   0.1 M Tris - 12.11 g

   0.1 M NaCl - 5.8 g

   5 mM $MgCl_2.6H_2O$ - 1.01 g

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

6 Substrate solution

   Solution A

   Nitro Blue tetrazolium (NBT) - 75 mg

   DMFA - 1 ml

   Solution B

   Bromo chloro Indolyl phosphate (BCIP) - 50 mg

   DMFA - 1 mg

APPENDIX IX

1. Stock solutions for MS basal medium

Sl. No.	Constituents	Amount of chemical taken (mg)	Amount mg g^{-1}	Solution ml l^{-1}
1.	Stock solution I (250 ml 10 X)			
	$MgSO_4$. 7 H_2O	3700	370	
	KH_2PO_4	1700	170	25 ml
	KNO_3	19000	1900	
	NH_4NO_3	16500	1650	
2.	Stock solution II (100 ml, 20 X)			
	$CaCl_2$. 2 H_2O	8800	440	5 ml
3.	Stock solution III (100 ml, 100 X)			
	H_3BO_3	620	6.2	
	$MnSO_4$.4 H_2O	1690	16.9	
	$ZnSO_4$. $2H_2O$	860	8.6	1 ml
	Na_2MoO_4. $2H_2O$	25	0.25	
	KI	83	0.83	

4.	*Stock solution IV (100 ml, 20 X) $FeSO_4$. 7 H_2O Na_2 EDTA. 2 H_2O	 556 746	 27.8 37.3	5 ml
5.	Stock solution V (250 ml-500 X) $CuSO_4$. 5 H_2O $CoCl_2$. 6 H_2O	 12.5 12.5	 0.025 0.025	.5 ml
6.	Stock solution VI (100 ml- 100 X) Thiamine HCl Pyridoxin HCl Nicotinic acid Glycine Myoinositol – 100 mg Sucrose – 30 g Agar – 7.5 g	 10 50 50 200	 0.1 0.5 0.5 2	1 ml

*Dissolve $FeSO_4$. 7 H_2O and Na_2 EDTA. 2 H_2O separately in 45 ml distilled water by heating and constant stirring. Mix the two solutions and adjust the pH to 5.5 and add distilled water to make up the final volume to 100 ml.

APPENDIX X

BBTV isolate

AAATCCATCAAGAAGAGGCGGGTTGGGCGCCGGAAGTATGGCAGCAA
GGCGGCAACGAGCCACGACTACTCGTCGTTAGGGTCAATATTGGTTCC
TGAAAACACCGTCAAGGTATTTCGGATTGAGCCTACTGATAAAACATT
ACCCAGATATTTTATCTGGAAAATGTTTATGCTTCTTGTGTGCAAGGTG
AAGCCCGGAAGAATACTTCATTGGGCTATGATTAAGAGTTCTTGGGAA
ATCAACCAGCCGACAACCTGTCTGGAACCCCCAGGTTTATTTATTAAA
CCTGAACATAGCCATCTGGTTAAACTGGTATGTAGTGGGGAACTTGAA
GCAGGAGTCGCAACAGGGACATCAGATGTTGAATGTCTTTTGAGGAA
GACAACCGTGTTGAGGAAGAATGTAACAGAGGTGGATTATTTATATTT

APPENDIX XI

BSV isolate

AAAAACAGAATCATTATTCGAGGAGTCAACGGAGTCACTGAAGTAAA
CGAGGTGACATCAGCGGGAAAGCTATGGGTTGGTAAGCAATGGTTCT
ACCTCCCTCAAACTTTTATTATGCCTTCATTAGCTGATGGAGTTCATAT
GATCATAGGCATGAATTTTATTAGAACTGTTGGCCTAAGGATAGAAAA
TGGTGAGGTCACAATTTATAAGATCATGACAACAGTACAAGCCCCACC
AATAGTTCATGAGCTGAATTATATTGATGAACTAGAACTGGAACTTCA
TGAATACTATAACATATGTGCAGCTGAGAGTTCTAGAGGGGAAATTTC
TGAAGAATTTATATCTCCTGACATTATTGGAAAAATGAAAAAATTGGG
ATATATTGGAGAAGAACCTCTCAAACATTGGGAGAAAAATCAGGTGA
AATGTAGGATTGAAGTAAAAAACCCTGATATGATTATTGAAGATAGGC
CATTAAAACATGTTACCCCTACAATGAAAGAAACCATGGCTAAGCATG
TCCAGAAGCTTTTAGAACTTAAAGTGATCAGGCCTTCAAGCTCAAAAC
ATAGAACAACGGCAATGATAGTAGAATCT

APPENDIX XII

CMV isolate

TGCCGGTCGTAACCGTCGACGTCGTCCGCGTCGCGGTTCCCGCTCCGC
CTCCTCCTCCGCGGATGCTACGTTTAGAGTCCTGTCGCAACAGCTTTCG
CGACTTAACAAGACATTAGCAGCTGGTCGTCCTACTATTAACCACCCA
ACCTTTGTGGGTAGTGAGCGTTGTAAACCTGGATACACGTTCACCTCG
ATTACCCTGAAGCCACCGAAAATAGACAAAGGGTCTTATTATGGCAAA
AGGTTGTTACTTCCTGATTCAGTCACTGAGTTCGATAAGAAGCTTGTTT
CGCGCATTCAAATTCGAGTTAATCCTTTGCCGAAATTTGATTCTACCGT
GTGGGTGACAGTCCGTAAAGTTCCTGCCTCCTCGGACCTGTCCGTTTCC
GCCATCTCTGCCATGTTTGCGGACGGAGCCTCACCGGTACTGGTTTATC
AGTATGCTGCATCTGGTGTTCAAGCCAACAACAAATTGTTGTATGATC
TTTCAGTGATGCGCGCTGATATTGGTGATATGAGAAAGTACGCCGTAC
TCGTGTATTCAAAAGACGATGCGCTCGAGACGGACGAACTAGTACTTC
ATGTCGACATTGAGCACCAACGCATTCCCAC

IMMUNOLOGICAL AND MOLECULAR DETECTION OF BANANA VIRUSES AND PRODUCTION OF DISEASE FREE PLANTING MATERIALS

by

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(2011-11-110)

Abstract of the thesis submitted in the partial fulfillment of the requirement for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture

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2014

ABSTRACT

The study entitled "Immunological and molecular detection of banana viruses and production of disease free planting materials" was conducted in College of Agriculture, Vellayani, and Thiruvananthapuram during the period of 2011-2014.

Symptomatological studies showed that the characteristics symptoms caused by BBTV were small, brittle leaves with thickened veins which remained bunched at the top of the pseudostem. Plants with early infection did not produce fruits, where plants with later infection produce bunch with reduced size, weight and mishapen fingers. The characteristic symptoms caused by BBrMV were reddish spindle shaped lesion in the pseudostem, flag leaf sheath, leaf petiole, and bract. Leaves of infected plants showed characteristic chlorotic spindle shaped lesion on the leaf lamina. The characteristic symptoms of BSV were chlorotic streaks in the leaf lamina. Later the chlorotic streaks became necrotic. The characteristic symptom of CMV was mosaic pattern in the leaf lamina.

The pathophysiological studies conducted in cultivar Nendran revealed that there was significant difference in carbohydrate, chlorophyll, protein and phenol content in infected plant when compared to healthy ones.. The activity of defence related enzymes like peroxidase, polyphenol oxidase and phenylalanine ammonialyase were found to be more in infected plants. Electrophoretic analysis of protein in virus infected samples through SDS-PAGE revealed the presence of an additional protein in the protein profile. The protein profile of BBTV infected sample showed one extra band with molecular weight of 20 kDa, BBrMV infected sample showed three additional protein band with molecular weight of 38 kDa, 29 kDa and 22 kDa, BSV infected sample showed three additional proteins with molecular weight of 25 kDa, 19 kDa, and 12 kDa, CMV infected sample showed one extra band with molecular weight of 25 kDa. Electrophoretic analysis of isozyme though native gel revealed the increased action of peroxidase enzyme in infected sample.

Detection of virus infecting banana was carried out using various immunological techniques such as DAC-ELISA and DIBA using polyclonal antiserum (Agdia) and monoclonal antiserum. Both the techniques were found to be efficient in detecting virus infecting banana. Molecular diagnosis of the BBTV was carried out using CP gene and replicase gene specific primers. PCR product with amplicon size of about 530 bp was observed for coat protein gene specific primer where 237 bp was observed for replicase gene specific primer. Molecular diagnosis of BSV was carried out using two CP gene specific primers resulted in PCR product with amplicon size of 664 bp and 730 bp. Molecular diagnosis of CMV was carried out using CP gene specific primer resulted in PCR product with an amplicon size of 687 bp. CP gene specific primer for BBrMV did not give positive result. Cluster dendrogram analysis revealed that the BBTV isolate was mostly related to BBTV coat protein gene of Burundi isolate. BSV isolate was mostly related to banana streak virus isolate Trichi, CMV isolate was mostly related to cucumber mosaic virus isolate Trichi coat protein gene.

The meristematic region of the virus infected banana suckers were excised and inoculated to MS media with BAP and NAA. The regeneration of plants from meristematic region was difficult because of high phenol production and contamination by endogenous bacteria. Meristem culture eliminated BBTV, CMV and BBrMV but not the BSV.

Based on the research result, the banana viruses can be detected using immunological and molecular technique and the meristem culture can eliminate all the banana viruses except BSV.