Summary and conclusions

*Musa* spp., is one of the important widely cultivating crops around the world. Ninety percent of *Musa* produced are immediately consumed, particularly in the poorest countries of Africa, Latin America and Asia (Foure and Tezenas du Montcel, 2000). According to FAO STAT 2008, India is in first place in world banana production. In India, major banana producing states are Maharastra, Tamil Nadu, Gujarat, Assam, Andhra Pradesh, Kerala and Karnataka. All the presently cultivating *Musa* spp., are hybrid varieties derived from two wild species i.e., *Musa acuminate* (AA genome) and *Musa balbisiana* (BB genome). Among several cultivars of banana, Dwarf Cavendish and Robusta are predominantly grown in India because of higher yields, resistance to strong winds and short cropping duration besides a good profit margin. In addition to these, other cultivars such as Poovan, Rasthali, Lalkela, Safed velchi and Karibale monthan are also grown.

Significant yield loss was reported in *Musa* spp. by several pests and pathogens across the world. The major pathogens like fungi, bacteria and viruses either singly or collectively cause severe diseases in *Musa* spp. Usually, mixed infections cause severe damage to the crop. Among the pathogen caused diseases, viral diseases are foremost important. This is because, fungal or bacterial diseases cause dramatic short term damage whereas the viral diseases are persistent and cause long term and severe damage often transmitted vertically through suckers to their progeny. In addition, no chemicals are so far available to control or eliminate viral diseases. However, stringent management practices limit viral attacks and thereby minimize their spread into new places. Correct identification of a pathogen in an infected plant is one of the major objectives to develop successful management practices. To date, eleven viruses have been reported to naturally
infect *Musa* spp., around the world. Among these viruses, banana bunchy top, banana streak, banana bract mosaic and cucumber mosaic viruses are important in several countries, including India, cultivating *Musa* spp.

*Banana bract mosaic virus*, a distinct species of the genus *Potyvirus*, causes bract mosaic disease in *Musa* spp. About 40 percent of yield loss due to this disease was reported in susceptible cultivars of *Musa* spp. In Kerala, maximum yield reduction recorded in Robusta was 70% followed by 52% in Nendran (Cherian et al., 2002). The virus is readily transmitted through vegetative propagules and by three aphid species. The infected plants show mosaic symptoms on leaves and inflorescence bract and purple coloured spindle shaped streaks on pseudostem of the infected *Musa* spp. The attempt to elucidate its etiology in India took longer time. Meanwhile the virus has been transmitted to several cultivars of *Musa* spp. Presently, it is a major constraint in southern states and requires quarantine restriction to limit its further spread to other major banana producing states of India (Selvirajan and Balasubramanian, 2008). Internal quarantine is necessary to prevent its spread into virus-free states. To develop stringent quarantine measures highly sensitive diagnostics are prerequisite to certify planting *Musa* spp. Preliminary characterization of the virus was already reported from Andhra Pradesh. Understanding, the genetic diversity of the virus isolates is important to develop genome based detection techniques, to know their evolution and to make use of viral genes for production of recombinant proteins and as transgenes.

*BSV*, a plant pararetrovirus, causes streak disease in *Musa* spp. BSV was reported from all countries of the world where the *Musa* spp., are grown predominantly. In India, the virus has been reported from Tamil Nadu, Kerala, Karnataka, Andhra Pradesh, West Bengal, Bihar, Assam, Maharashtra and Gujarat (Selvirajan et al., 1997). BSV infection results in significant yield losses ranging from 6 % to 90 % (Daniells et al., 2001). In India, about 48 % yield loss in cultivar Poovan was reported (Thangavelu et al., 2000). The virus is serologically and genomically highly heterogeneous. Based on these variations, the virus has been typified into different species like *Banana streak Mysore virus* (BSMyV, a *Badnavirus*), *Banana streak Gold finger virus* (BSGFV, a *Badnavirus*), and *Banana streak Obino l’Ewai virus* (BSOLV, a *Badnavirus*). All these viruses cause
similar chlorotic or necrotic streaks or stripes on the infected *Musa* spp. Poovan, one of the important cultivars grown in India, is almost 100 percent infected (Selvirajan and Balasubramanian, 2008). It is not too easy to develop successful serological or genome based diagnostics to these viruses as they are serologically and genomically highly heterogeneous. Yet immunocapture and direct binding PCR s have been developed to detect BSV in *Musa* spp., by using a set of *Badnavirus* degenerate primers. In India, PCR based method was developed for BSV detection using primers specific to conserved domains of genome of *Badnavirus* (Cherian et al., 2004). The genome sequences of two BSV species (BSMyV and BSOLV) occurring in India have been generated. So far the genome sequences of BSV isolates infecting *Musa* spp., in Andhra Pradesh are not available for their precise discrimination and for development of genome based detection tests.

*Cucumber mosaic virus*, a type species of *Cucumovirus* genus, causes mosaic or infectious chlorosis disease in *Musa* spp. In India, this disease was identified in early 1940s in Maharastra. Later the disease has been reported from many parts of the country (Rao, 1980). In Kerala state, about 45 % to 62 % yield loss was reported by CMV infection. The observations recorded during the survey on CMV incidence in banana fields of 20 villages of Nanded and Parbhani districts of Marathwada region ranged from 15% to 27.5 %. In China, CMV incidence in some banana fields was from 40% to 100% Immunodiagnosics and nucleic acid based tests for detection of CMV infecting *Musa* spp., in Andhra Pradesh have already been developed. In India, partial genome sequences of several isolates of CMV form different hosts have been reported. But the information on genome sequences of CMV isolates infecting *Musa* spp., is scanty. The generation of sequence data for CMV isolates naturally infecting *Musa* spp., as well as other crops and weeds growing in and around *Musa* plantations in Andhra Pradesh may help to understand the genetic relatedness among the isolates and with other reported isolates and to develop specific genome based diagnostic tests.

In the present study, partial genome sequences were generated for BSV, BBrMV and CMV isolates naturally infecting *Musa* spp., and to CMV isolates infecting other crops in Andhra Pradesh to know their genetic variability and exploited the CP gene of
BBrMV to produce PAbs for development of immunodiagnositics for its detection. Further, generated genome sequences were used to design virus specific primers and developed multiplex PCR for their parallel detection.

**Reasons for selecting the present problem**

- Greater economic loss was reported in *Musa* spp., due to BSV, BBrMV and CMV infections in India.
- Limited information on diversity of the genomes of viruses naturally infecting *Musa* spp., in India is available. Information on sequences of virus genomes is necessary to develop molecular diagnostics and to exploit viral genes.
- Reliable diagnostics, prerequisite to develop successful management practices have not been developed for indigenous virus isolates.
- Symptom based diagnostics is not reliable as CMV and BSV cause similar symptoms on *Musa* spp.
- ELISA based tests are reliable, inexpensive and suitable for large scale application if quality antibodies are available. To produce quality polyclonal antibodies to a virus, purified virus is required. But it is an uphill task to purify the virus from *Musa* spp. directly.
- Attempts so far have not been made to produce recombinant DNA technology based PAbs to viruses infecting *Musa* spp., in India;
- Genome based tests like RT-PCR, IC-RT-PCR and multiplex-PCR are highly sensitive, rapid and most reliable to detect viruses in infected plants. To develop such techniques for viruses occurring in a given geographical area, their genome sequence data are prerequisite.
Findings of the present study

Partial genome sequence analysis of BBrMV, CMV and BSV

The suckers of *Musa* spp., suspected for BBrMV, CMV and BSV infections were collected from different districts of Andhra Pradesh state and propagated in the garden of Virology Department, S.V. University, Tirupati.

Other host samples like brinjal, chilli, tomato and tagetus suspected for CMV were collected around Tirupati and from near by villages for PCR amplification and sequencing.

Samples suspected for BBrMV were screened by DAC-ELISA using heterologous TEV antiserum and the positive samples were properly labeled and used for further experiments.

Total RNA was isolated from infected BBrMV and CMV suspected and corresponding healthy *Musa* spp., leaf samples by Qiagen RNeasy mini kit method. Similarly total RNA was isolated from brinjal, chilli, tagetus and tomato host leaf samples suspected for CMV.

Oligo dT and sequence specific reverse primers designed were used for the synthesis of first strand cDNA to BBrMV and CMV, respectively.

The CP gene of six BBrMV isolates was amplified by using sequence specific primers, and the size of amplificons was estimated as ~900 bp on agarose gel.

The CP gene of four CMV isolates viz., 2 isolates from *Musa* spp., and one isolate from each of brinjal and chilli was amplified by RT-PCR, and the amplicons size was ~650 bp.

Similarly, the MP gene of seven CMV isolates viz., 2 isolates from *Musa* spp., two from brinjal and one isolate from each of chilli, tomato and tagetus were amplified by RT-PCR, and amplicons size determined as ~830 bp on agarose gel.
Total DNA was isolated from BSV suspected *Musa* spp., leaf samples and the part of RT/RNaseH region of the virus was amplified in 8 samples by using degenerate primers. The amplified product is ~590 bp.

The amplicons of three viruses were gel extracted by Qiagen gel extraction kit method and cloned separately in to a T tailed vector i.e., pGEMT easy / pTZ57R/T.

The ligated products of the respective viruses were transformed into *E.coli* (*DH5a*) cells, and the transformants were screened by blue and white colony screening method.

White colonies were picked up and the isolated recombinant plasmid DNA used for restriction digestion analysis (insert release). PCR amplification of cloned inserts was also carried for further confirmation.

For BBrMV, the amplified CP gene of six isolates was sequenced. Each isolate CP gene is 900 nucleotides in length and deduced amino acids are 300 residues.

The CP gene sequences of 6 BBrMV isolates from Andhra Pradesh (AP1 to AP6) were compared with the CP gene sequences of other BBrMV isolates originated from India and other countries. The sequence comparison revealed that they have 94 to 99 and 95 to 99 percent similarity with other isolates at the CP gene nucleotide and CP amino acid levels, respectively. The variability among AP isolates was not more than 2 percent at both CP gene nucleotide and CP amino acid levels.

In all the six BBrMV AP isolates, a DAG triplet at N terminal region and AFDF, RQ at C-terminal region of the CP amino sequences were observed. Like in other potyviruses, the WCIEN box was observed in the core region of the AP 2 and AP6 isolates but not in remaining the four viz., AP1, AP3, AP4 and AP5 isolates, where in the isoleucine is replaced by the valine in the WCIEN box.
Phylogenetic analyses were made based on the results of multiple alignments of CP gene nucleotide and CP amino acid sequences of BBrMV. It revealed that BBrMV AP1 to AP5 isolates grouped under one clade and they clustered very close to Indian isolates viz., BBrMV-Coimbatore, -Karnataka and Indian isolate 12. Whereas the BBrMV AP6 isolate grouped with BBrMV-Cd (cardamom, Indian isolate), BBrMV-Tri and other foreign isolates.

The RT/RNase H gene region of 8 BSV isolates (BSV-AP 1 to BSV-AP 8) has been sequenced and the resulted sequence is 589 to 597 nucleotides in length.

The sequences of eight BSV isolates were aligned with 31 reference sequences of different BSV isolates. The results of alignments revealed that the isolates from AP1 to AP4 showed 99 percent sequence similarity with BSMyV at amino acid levels. The same four BSV isolates from Andhra Pradesh showed 25 to 36 percent variation with other compared BSV isolates at amino acid levels. In phylogenetic tree the four virus isolates clustered with BSMyV.

Similarly, BSV-AP5 and -AP6 were grouped with Musa acuminata endogenous badnavirus isolates and showed 85 to 99 percent similarity at amino acid levels.

The BSV-AP7 and -AP8 grouped with Musa baltisiana endogenous badnavirus isolates and Banana streak Obino l'Ewai virus isolates and showed 85 to 97 percent and 98 percent similarity, respectively at amino acid levels.

The CP gene of CMV isolates from Musa spp., (2), brinjal (1) and chilli (1) were sequenced. The CP gene of four isolates is 657 nucleotides in length and deduced amino acid residues are 218. Similarly, the MP gene sequences of seven isolates were generated. The MP gene of 7 CMV isolates is 830 nucleotides in length and the deduced amino acids are 276.

The comparative sequence analyses of CP gene of four CMV isolates showed 75 to 76 percent similarity at nucleotide levels and 78 to 82 percent sequence similarity at amino acid levels with subgroup II CMV isolates. Whereas with
subgroup I isolates, the observed similarity was 90 to 99 percent and 93 to 99 percent at nucleotide and amino acid levels, respectively.

- The results of multiple alignments based on amino acid sequence of CP were used to generate phylogenetic tree. The tree elucidated that the four CMV isolates grouped along with subgroup 1B CMV isolates.

- The MP gene sequence of the present study seven CMV isolates compared with 27 other sequences of CMV isolates (GenBank sequences) of various hosts from different countries around the world. The percent identity of seven CMV AP isolates was 78 to 79 percent and 82 to 85 percent at nucleotide and amino acid levels, respectively with subgroup II whereas with subgroup I, 89 to 99.6 and 92-99.7 percent at nucleotide and amino acid levels, respectively. It indicates the present studying seven CMV isolates belongs to the subgroup I.

- In the phylogenetic analysis based on MP gene nucleotide and amino acid sequences, among the seven CMV-MP isolates of the present study, CMV-B1, B2 and -Tagetus were grouped as a cluster and the remaining four i.e., CMV-Chilli, -Tom, -Br1 and -Br2 grouped into another cluster. A cluster having CMV-banana isolates from Kerala, Uttar Pradesh, Maharastra and Lucknow is close to the cluster that contains CMV-banana isolates from Andhra Pradesh. Similarly, the cluster having CMV-Chilli, -Tom, -Br1, -Tagetus and -Br2 is close to the cluster containing CMV-Jatropha and -Amaranthus, isolates from Lucknow.

Cloning and expression of CP gene of BBrMV in E.coli and production of polyclonal antiserum to rCP.

- The CP gene of BBrMV amplified with newly designed sequence specific primers containing BamHI and EcoRI enzymes sites at 5' and 3' regions, respectively. The amplified CP gene product was cloned into pGEMTeasy cloning vector

- The insert released from the vector by BamHI and EcoRI enzymes digestion was gel extracted and cloned into pRSET A expression vector.
The ligated product was transformed into *E. coli* BL21 (DE3) pLys S cells and the potent recombinant colony was confirmed by restriction enzyme digestion (*BamH*I and *EcoRI*) analysis, PCR amplification and sequencing of plasmid DNA.

Recombinant plasmid DNA containing *E. coli* BL21 (DE3) pLys S cells were induced with 0.4 mM IPTG and incubation at 25°C temperature for expression of CP of BBrMV in soluble form.

The expressed CP was purified by Ni-NTA affinity column and its molecular weight was determined as 38 kDa by 12% SDS-PAGE analysis.

The expressed protein in western blot analysis reacted with heterologous potyvirus antisera of TEV and WMV.

Polyclonal antibodies were produced by injecting the expressed purified recombinant CP in to New zealand white rabbits. After a booster dose, 5 bleeds were collected from the ear vein of the rabbit.

The resulted polyclonal antiserum was titrated by DAC-ELISA using healthy and BBrMV infected *Musa* spp., leaf extracts as antigens. Among the five bleeds, fourth bleed found to be more specific with high titre than the remaining bleeds. The antiserum was preserved in aliquots by incorporating sodium azaide.

**Development of diagnostics for detection of BBrMV**

DAC-ELISA was optimized to detect BBrMV in *Musa* spp., using PAbs produced to rCP of BBrMV. In DAC-ELISA, PAbs (1: 5000, v/v dilution) detected the virus antigen in leaf extracts upto 1:500 dilution.

The produced PAbs to rCP of BBrMV also detected BBrMV in *Musa* spp., leaf extract by electro blot immuno assay.

For detection of BBrMV in *Musa* spp., by RT-PCR, the total RNA made into dilutions viz., 1:10, 1: 50, 1:100, 1:500, 1:1000, 1:5000 and 1:10,000 (v/v) was
used as source of templates for PCR amplification. The positive amplification signal was observed up to 1:5000 dilution.

- In IC-RT-PCR, BBrMV was detected up to 1:10,000 dilutions in *Musa* spp., leaf extracts.

- The comparison of the sensitivity of DAC-ELISA, RT-PCR and IC-RT-PCR for detection of BBrMV in *Musa* spp., revealed that the IC-RT-PCR is 20 - and 2 - times more sensitive as compared to ELISA and RT-PCR, respectively.

**Development and optimization of multiplex PCR for parallel detection of three viruses in *Musa* spp.**

- The partial genome sequence analyses of BBrMV, BSV and CMV isolates enabled to design primers for amplification of selected portions of specific viral genomes by multiplex PCR.

- The part of CP (389 bp), RT/RNaseH (597 bp) and MP (831 bp) genes of BBrMV, BSV and CMV, respectively were successfully amplified in infected samples of *Musa* spp. This test has potential application in screening and certification of these three viruses-free *Musa* spp. planting material, a prime approach in disease management.

- This test may save costs, labour and testing time as compared to detection of three viruses individually.

**Future perspectives**

- A comprehensive data on the distribution and genetic diversity of BBrMV, BSV and CMV isolates of *Musa* spp., across India is required to identify genetic variants, if any, and to develop successful detection methods for use in quarantine.

- To apply widely used ELISA based tests, there is a need to produce high quality antibodies by recombinant DNA technology approach for detection
of BSV in *Musa* spp. in India. Quality antibodies are essential to detect episomal form of BSV.

- The mystery behind the activation of BSV endosomal forms during micropropagation is to be resolved for preventing the rapid spread and distribution of virus through propagules of *Musa* spp.

- The highly sensitive immunocapture-multiplex-PCR (IC-M-PCR) for parallel detection of all viruses naturally infecting *Musa* spp., is to be developed in India. For this, antibodies for targeted viruses are mandatory and need to be produced.

- Sensitive diagnostics could be useful to identify alternate natural hosts, if any, especially for BSV and BBrMV.

- The sequenced CP genes of BBrMV and CMV isolates of *Musa* spp., can be exploited as transgenes in the development of transgenic *Musa* spp. Infact BBrMV CP clone was spared to Dr.T.R.Ganapathi, Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Mumbai for this purpose.