INTRODUCTION

Banana and plantain (Musa spp., ) are grown in more than 125 countries and provide hundreds of millions of people throughout the tropics and subtropics with an essential staple food and account for one of the most widely exported fruit in the world (Hu et al., 2007). According to FAO (2008), the production of banana around the world is 9, 07, 05,922 tonnes, and India occupies the first place in the world banana production (2, 32, 04,800 tonnes). In India, major banana producing states are Maharashtra, Tamil Nadu, Gujarat, Assam, Andhra Pradesh, Kerala and Karnataka. The genus Musa has 35 species and about 100 subspecies. Cultivated banana and plantain originated mainly from two wild species, Musa acuminata (AA genome) and Musa balbisiana (BB genome) resulting in a series of diploid, triploid and tetraploid genomes (AA, BB, AAB, ABB, AAAB, ABBB). 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 velshi and Karibale monthan are also grown.

Due to intensification of crop production during the past 20 years, an increasing number of new pests and diseases have been identified that can cause a significant reduction in yield. A major problem of plantain and banana landraces is their susceptibility to various fungal (e.g.,black sigatoka, Fusarium wilt), viral (e.g.,banana bunchy top, banana streak, cucumber mosaic and bract mosaic viruses), bacterial (e.g., bacterial wilt, bacterial soft rot), nematodal and insect attacks. If unchecked, all of these
cause leaf decay, thereby reducing the photosynthetic area, and causing a reduction in yield.

So far, eleven viruses viz., *Banana buch top virus* (BBTV, a *Babuvirus*), *Banana streak Mysore virus* (BSMysV, a *Badnavirus*), *Banana streak Gold finger virus* (BSGFV, a *Badnavirus*), *Banana streak Obino l’Ewai virus* (BSOLV, a *Badnavirus*), *Banana bract mosaic virus* (BBrMV, a *Potyvirus*), *Banana mild mosaic virus* (BanMMV, an unassigned member of the *Flexiviridae*), *Banana virus X* (BVX, a new unassigned species in the family *Flexiviridae*), *Cucumber mosaic virus* (CMV, a *Cucumovirus*), *Abaca mosaic virus* (AbaMV, a *Potyvirus*), *Tobacco mosaic virus* (TMV, a *Tobamovirus*) and *Banana die-back virus* (BDBV, a probable *Nepovirus*) were reported to naturally infect *Musa* sp., in different parts of the world. In India, BBTV, BSMysV, BBrMV and CMV are the major constraints to *Musa* spp., cultivation.

1.1 Background for the present work

In India, several cultivars of *Musa* spp., are widely grown through vegetative propagation and exporting to several countries around the world. Recently, banana plants generated by tissue culture technology are widely used for cultivation but this technology is not suitable to all cultivars. In such circumstances, vegetative propagation is only the option for their cultivation. Viruses reported so far in banana are primarily transmitting through vegetative planting materials like suckers, bits and corms and have a direct or indirect effect on production by reducing the plant growth and yield. However, few viruses like CMV, BBrMV, and BSV have insect vectors to horizontally transmit them from infected to healthy plants.

Viral infections can not be eliminated by chemical or by any other treatments, which means that control of viral diseases must be based on preventing infection rather than curing the disease (Hull, 2002). To develop successful preventing methods for viral diseases in *Musa* spp., it is ideal to know about the exact identity of causal agent(s) of the disease(s). Symptom based detection is not worth-while in banana, because the plants sometimes do not express any symptoms and some times the symptoms due to different
viruses are very similar. For example, BBrMV, CMV and BSV cause similar chlorotic streak mosaic symptoms on banana leaves. Symptoms of banana mosaic can also be misidentified as zinc deficiency and the genetic abnormality. Serological methods like ELISA and ISEM with conventional antibodies are insensitive and expensive for the detection of viruses in *Musa* samples, respectively. Methods based on nucleic acid hybridization have been replaced by PCR amplification, which is a less cumbersome, sensitive and reliable technique for detection of viruses in *Musa* spp. In many advanced banana producing countries, techniques like RT-PCR, IC-RT-PCR and multiplex-PCR were developed based on the PCR amplification and are being widely used in indexing and certification of planting materials of *Musa* spp., and hence better management of viral infections.

Information on genetic diversity among isolates of a given virus occurring in a specific geographic region is important to understand its phylogenetic relationships with similar viruses of the same or different regions (Hull, 2002). Further, the information could be utilized in the exploitation of viral genes or sequences for varied purposes (transgenics, overexpression of specific genes in bacterial hosts, designing of primers and probes).

For large scale screening of plant viruses, ELISA is more convenient than nucleic acid based tests (Webster, 2004). ELISA is one of the sensitive and reliable diagnostic techniques if the antibodies are highly specific to virus under testing. Quality of antisera depends on the purity of the virus antigen preparations. Therefore, application of this technique for indexing BBrMV helps to select virus-free planting material and thereby minimize the spread of virus into new geographical areas. No alternative hosts for BBrMV outside *Musa* genus are so far known, and the virus has not been transmitted by mechanical inoculation. Therefore, the purification of this virus directly from *Musa* spp., is very difficult and thus a major hurdle in the production of good quality antiserum necessary for development of immunoassays like ELISA and IC-RT PCR for its detection. The recombinant DNA technology has been applied in plant virology to circumvent this difficulty. The coat protein or nucleocapsid gene of a target virus is overexpressed in bacterial or yeast systems, expressed protein is purified and used as an
antigen to produce high quality antibodies. Recently, this approach has gained momentum in the production of high titered antibodies to plant viruses (Sreenivasulu et al., 2009).

In India, exchanging of *Musa* spp., planting material from one state to another is quite common. Movement of planting material without indexing and certification would allow the spread of viruses into new places and thereby cause severe damage to the crop. Significant damage was reported in *Musa* spp., by BBrMV, BSV and CMV in major banana producing states in India. To minimize the spread of viral diseases through planting materials, introduction and implementation of new technology is compulsory. Therefore, the present study is aimed to analyze partial genome sequences of different isolates of BBrMV, BSV and CMV infecting *Musa* spp., in Andhra Pradesh state, India and to develop serological and molecular diagnostics for these viruses.

1.2 Banana bract mosaic

The disease banana bract mosaic first noted in 1979 in Philippines has been reported to cause yield loss up to 40 percent in highly susceptible varieties, particularly in ABB cooking banana cultivars ‘Saba’ and ‘Cardaba’ (Espino et al., 1990). Subsequently, it was reported from other countries like Sri Lanka, Australia and India (Rodoni et al., 1997; Thomas et al., 1997). The causal agent of this disease is *Banana bract mosaic virus* (BBrMV) that belongs to the genus *Potyvirus*, family *Potyviridae*. It has non-enveloped flexuous filamentous virions, each 750 nm X 11 nm in length. Banana bract mosaic was named based on the conspicuous dark purple streaks on the inflorescence bracts, purple colored spindle shaped streaks on petioles and pseudostem, and occasional interveinal chlorotic streaks on leaves of banana.

Recently, Ha et al., (2008) reported the complete genome sequence of BBrMV (Philippine isolate). Earlier, partial genome sequences of several isolates of BBrMV from different banana producing countries were reported (Rodoni et al., 1999). Among them, most of the sequences covered 3' terminal region of the genome i.e., coat protein (CP) and untranslated region (UTR). These two regions are commonly used as markers of
genetic relatedness of potyviruses. Rodoni et al., (1999) sequenced the entire CP and part of 3'UTR of nine different isolates of BBrMV from five different countries. The sequence variability among these isolates ranged between 0.3% and 5.6%, and 0.3% and 4.3% at nucleotide and amino acid levels, respectively. In India, very limited genome sequence data for BBrMV are available (Ramesh et al., 2006; Rodoni et al., 1999). These isolates with other isolates reported from different countries showed 1.2% to 5.6% and 2% to 4% sequence variation at nucleotide and amino acid levels, respectively. In Andhra Pradesh, cultivation of certain banana cultivars is severely affected by this disease. This is due to lack of certified virus-free planting material for further cultivation. Information on genetic diversity of the virus is more important to know the strain(s)/isolate(s) of the virus existing and to develop serological/molecular diagnostics for indexing and certification of *Musa* spp. To date, no such type of work has been carried out in the state of Andhra Pradesh.

In the present study, partial genome sequence analysis of different isolates of BBrMV in Andhra Pradesh state was carried out to know their diversity and to produce polyclonal antibodies to rCP of BBrMV with a aim to develop sensitive diagnostics like ELISA and IC-RT-PCR for its detection in *Musa* spp.

### 1.3 Banana streak

Banana streak disease was first reported in Morocco (Lockhart, 1993) and later it has been reported in nearly all countries where this crop is grown (Jaufeerally-Fakim et al., 2006). BSV, the causal agent of this disease, is a member of *Badnavirus* genus, family *Caulimoviridae*. The virus has a circular, non-covalently closed dsDNA genome of 7.4 kbp encapsulated in non-enveloped bacilliform particles (Harper et al., 2004; Agindotan et al., 2006). Characteristic symptoms of BSV infection in banana plants are continuous or discontinuous interveinal chlorotic or necrotic streaks on the leaves, stunting of diseased plants and occasionally heart rot of the pseudostem and plant death. In the green houses, BSV is typically transmitted by mealybug (*Planococcus citri*). BSV infection induces yield losses and restricts the international exchange of banana germplasm. Pronounced antigenic as well as molecular variability among BSV isolates
was reported (Lheureux et al., 2007). The virus can occur in two main forms within the host, firstly integrated into the host genome (endogenous BSV) and secondly an episomal form. Recent reports indicate that BSV infection may arise from the activation of viral sequences that are integrated into the *Musa* genome causing problems not only in the safe movement of germplasm, but also in breeding and micropropagation of *Musa* spp. The symptoms of BSV may sometimes be very similar to that of CMV infection. Reliable detection of BSV is necessary because of the movement of *Musa*, mainly in the form of tissue culture plants, around the globe.

During the last 10 years, number of reports across the world on BSV published. It is genomically and serologically heterogenous worldwide. Several partial and a few full length genome sequences of the virus were generated. Recently, based on the genetic variation, the causal virus discriminated into different species of the genus *Badnavirus*. Virus isolates show more similarity within the species and more than 30% variation between the species. The differences greater than 20% across the RT region indicated different species. Based upon this criteria, 12 new species of BSV infecting Ugandan banana were suggested (Harper et al., 2004). In India, banana streak is more prevalent in several banana producing states. Very few sequences of BSV are available from India. Recently, Selvirajan et al., (2008) have completely sequenced the BSMyV and BSOLV from cultivar Mysore in India. They showed very close similarities with the previously reported BSMyV (NC 006955) and BSOLV (NC 003381) sequences, respectively. To know genetic diversity of BSV in India, more number of isolates of this virus need to be sequenced. So far no reports from the state of Andhra Pradesh even though the disease is prevalent in certain cultivars of *Musa* spp.

In the present study, degenerated primers specific for part of reverse transcriptase/RNaseH genes of the virus are used to study the genetic diversity of the BSV species infecting different *Musa* spp., in Andhra Pradesh state and to develop multiplex-PCR for parallel detection of this virus along with BBrMV and CMV.
1.4 Banana mosaic/infectious chlorosis

Banana mosaic/infectious chlorosis caused by CMV is one of the most serious virus diseases of banana in many tropical and sub-tropical countries. It was first reported in New South Wales in 1929 and in Central America in 1957. Subsequently, it was reported from many countries wherever *Musa* spp., are grown. This aphid-transmitted virus can be of economic importance especially when heart rot symptoms occur (Bouhinda and Lockhart, 1990). This disease is characterized by a conspicuous interveinal chlorosis of leaves. Young plants raised from suckers derived from diseased plants show interveinal chlorosis from an early age. As the disease progresses, emerging leaves misshapen and leaf margins are wavy in appearance. In Kerala, CMV infection resulted in 45 to 62% reduction in bunch weight (Hema et al., 2000). Severe strains of CMV produce more pronounced symptoms, which can include necrosis of emerging cigar leaves, leading to varying degrees of necrosis in the unfurled leaf lamina (Hema et al., 2000). These symptoms are often confused with those caused by BSV (Provost et al., 2006).

Numerous strains of CMV have been classified into two major sub-groups (subgroup I and II) on the basis of serological properties and nucleotide sequence homology. An array of both partial and full length sequences is available for CMV around the world due to its broad host range. Based on the sequence similarity, isolates infecting *Musa* spp., were assigned to the subgroup I. CMV isolates from Western Australia, infecting *Musa* spp., belong to subgroup I. Sequence analysis of these isolates with other reported isolates revealed 98% homology to sequences of CMV subgroup I isolates and 76% homology to sequences of CMV subgroup II isolates. In India, several isolates of CMV sequences were reported from different hosts. But the information on genome sequences of CMV isolates infecting *Musa* spp., is very limited. It was found that the sequences of isolates of CMV isolated from banana and other hosts in India belong to the subgroup I.

The present study is focused on determining the genetic diversity of CMV isolates infecting *Musa* sp., and other hosts and to develop multiplex-PCR for simultaneous detection of CMV, BBrMV and BSV in *Musa* spp.
1.5 Objectives of the present study

1. To collect and maintain isolates of BBrMV, CMV and BSV infecting Musa spp., in Andhra Pradesh state.

2. To isolate total RNA or DNA from infected plant samples and to synthesize first strand cDNA for BBrMV and CMV using oligo-T or virus specific primers.

3. To amplify CP gene of BBrMV, CP and MP genes of CMV and part of RT/RNaseH genes of BSV using virus specific primers.

4. To clone, sequence and analyse genetic variability of PCR amplicons of above 3 viruses.

5. To clone and express the CP gene of BBrMV in E.coli.

6. To purify the expressed recombinant coat protein of BBrMV by Ni-NTA columns and to produce polyclonal antiserum against rCP in New Zealand white rabbit.

7. To optimize antibody based tests for BBrMV detection in Musa spp.

8. To develop multiplex-PCR for parallel detection of BBrMV, BSV and CMV in the samples of Musa spp.
1.6 Work plan

1.6.1 Partial genome sequence analysis BBrMV, BSV and CMV

\[\text{Musa species}\]

Isolation of total RNA/DNA

\[\text{RT}\]

First strand cDNA
(For CP gene of BBrMV and CMV; MP gene of CMV)

DNA
(For RT/RNase H gene)

\[\text{PCR amplification}\]

\[\text{TA cloning into pGEMTeasy/pTZ5R/T}\]

\[\text{Transformation}\]
\[\text{Screening (blue/white colony)}\]
\[\text{Confirmation (Restriction enzyme digestion and PCR amplification)}\]

\[\text{Sequencing}\]

\[\text{Sequence analysis}\]
1.6.2 Cloning and expression of BBrMV and production of PAbs to rCP of BBrMV.

- PCR amplification of CP gene of BBrMV
- Directional cloning into pRSET A bacterial expression vector
- Transformation into BL21 (DE3)pLys S bacterial cells
- Confirmation (Restriction enzyme Digestion, PCR amplification and sequencing)
- Overexpression in BL21 (DE3)pLys S bacterial cells
- Purification of rCP of BBrMV by Ni-NTA affinity columns
- Immunization of rCP of BBrMV in to New Zealand white rabbits
- Production of PAbs to rCP of BBrMV

1.6.3 Diagnostics

- DAC-ELISA
- Electro immunoblot assay
- RT-PCR
- IC-RT-PCR

\{ for detection of BBrMV in Musa spp \}

- Multiplex PCR for detection of BBrMV, BSV and CMV in Musa spp