6.1 Introduction

For most studies in plant virology, it is essential to be able to establish a proper diagnosis, i.e., to know how to recognize a disease and identify precisely the causal virus or viruses. For successful implementation of control programs for viruses propagated through vegetative plant parts, it is necessary to have rapid, reliable sensitive tests to screen plant propagules in quarantine, tissue culture generated plants by companies and to determine the distribution of the virus in commercial fields (Hull, 2002). The ideal technique would be sensitive, inexpensive, easy to use and reliable. Several diagnostic techniques have been established based on serology and molecular biology based approaches (Webster, 2004; Rao and Singh, 2008).

Based on antigen and antibody interactions, several immunodiagnostic techniques are now available and widely used for the detection of virus(es) in suspecting host plants and insect vectors (Webster, 2004). Among these, ELISA, western blot and IC-RT-PCR have gained momentum in the field of plant virology. Of these, ELISA is widely used to screen samples on a large scale. However, every technique has its own advantages and limitations. For example, ELISA is an inexpensive and easy to handle test but in terms of sensitivity and reliability towards the antigen again it depends on the quality of antiserum. The quality of antiserum in turn depends on the purity of virus antigen. If the antigen purity is high, the resulted antiserum is more specific and reliable. The quality conventional antibodies are readily available for several potyviruses that are easy to purify from their host plants. The recombinant DNA technology has been applied to generate antibodies to certain potyviruses having difficulties in purification from their...
host plants. For example, successful polyclonal antibodies and ScFv antibodies were produced to *Sugarcane streak mosaic virus* and *Potato virus Y*, respectively (Hema et al., 2003; Singh, 2000).

Different ELISA based techniques have been developed for the detection of BBrMV in *Musa* spp. For example, DAC-, DAS- and TAS-ELISAs have been used for the detection of BBrMV using either homologous or heterologous PAbs and homologous MAbs in The Philippines, Australia and India (Espino et al., 1990; Thomas et al., 1997; Rodoni et al., 1999 and Kiranmai et al., 2005). Serological techniques like chloroplast agglutination, micro precipitin, agar gel double diffusion, DAC-ELISA and dot immunobinding assay (DIBA) tests were used for the detection of BBrMV in *Musa* spp., in South India. (Selvarajan et al., 2006; Dhanya et al., 2007).

Sometimes serological techniques failed to detect existed virus in an asymptomatic plants due to less virus concentration and low sensitivity of the technique used. For example, serological techniques like ELISA, electroblot immunoassay and immunoelectron microscopy detected viral antigens up to 10 ng level whereas RT-PCR and IC-RT-PCR detected the same antigen up to 1 ng level. This indicates clearly that molecular techniques are relatively more sensitive when compared to serological methods. But molecular techniques have the limitation for large scale screening of samples due to high cost.

Reverse transcription - PCR (RT-PCR), a novel technique, has been applied for detection of several potyviruses around the world (Rao and Singh, 2008). An RT-PCR assay is able to reliably detect one infected seed in one thousand healthy seeds (Wylie et al., 1993). Several plant RNA viruses were successfully detected and identified in various asymptomatic hosts by using RT-PCR (Rao and Singh, 2008). Application of RT-PCR with degenerate primers, picked up several unknown plant viruses of different genera. RT-PCR using degenerate primers (designed based on specific conserved domains in virus proteins such as CP, heat shock-like protein or viral polymerase) has been used for the detection of several plant virus species that belong to the same genus or family (Langeveld et al., 1991 and Karasev et al., 1994). Two pairs of degenerate primers were
designed from sequences within the potyviral CI and HC-Pro coding regions, and these were shown to be more specific to members of the genus *Potyvirus* (Ha et al., 2008). The 3'-terminal genome regions of 21 viruses in the family *Potyviridae* have been amplified by RT-PCR using a universal primer designed to the conserved sequence GNNSQGP of NlB region of virus species of the family *Potyviridae* (Chen et al., 2001).

IC-RT-PCR avoids the interference of several host constituents that hamper the amplification of virus genome. In addition, this technique does not require time taking RNA preparations in the detection of plant viruses. Therefore, it is one of the preferred techniques to detect the virus in suspected plant materials.

For detection of viruses (CMV, BSV, BBrMV and BBTV) infecting *Musa* spp., techniques like ELISA, dot-ELISA, direct-PCR, immuno-capture-PCR, RT-PCR, IC-RT-PCR and multiplex-PCR have been used for detection of above viruses in different countries (Table 6.1).

Table 6.1: Tests applied for detection of certain viruses infecting *Musa* spp.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Antibody based tests</th>
<th>Virus genome based tests</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>BETV</td>
<td>DAS-ELISA and TAS-ELISA</td>
<td>PCR; IC-PCR; Nucleic acid hybridization</td>
<td>Gosling and Thomas, 1995; Xu and Ha, 1995; Arents et al., 1996; Thomas et al., 1997</td>
</tr>
<tr>
<td>BBrMV</td>
<td>DAS-ELISA, Dot-ELISA</td>
<td>RT-PCR; IC-RT-PCR</td>
<td>Rodini et al., 1999; Thomas et al., 1996</td>
</tr>
<tr>
<td>BSV</td>
<td>ELISA, ELISA, ISEM</td>
<td>PCR and IC-PCR</td>
<td>Lockhart and Glaszownd, 1993; Agindoyin et al., 2003; Harper et al., 2004</td>
</tr>
<tr>
<td>CMV</td>
<td>Dot-blot, ELISAs</td>
<td>RT-PCR; Nucleic acid hybridization</td>
<td>Hu et al., 1995; Kiranmai et al., 1995, 1996</td>
</tr>
</tbody>
</table>

Antibody based diagnostics using homologous antibodies for detection of BBrMV isolates infecting *Musa* spp., in India have not so far been developed. Hence, in the present study DAC-ELISA, electroblot immuno assay and IC-RT PCR techniques are developed using the PAbs produced against purified rCP and compared their relative sensitivities in detection of BBrMV infections in *Musa* spp. In addition, a multiplex-PCR technique was developed for parallel detection of BBrMV, BSV and CMV in *Musa* spp.
In multiplex-PCR, two or more primer sets designed for amplification of different targets, are included in the same reaction mixture. Primers used for multiplex PCR must be carefully designed so that they have similar annealing temperatures and lack complementarity.

6.2 Results and discussion

6.2.1 DAC-ELISA

DAC-ELISA is more versatile as the test can be automated and commercialized for application to crops like banana. In the present study, PAbs to rCP were used for the detection of BBrMV in infected Musa spp. The leaf extracts of healthy and infected Musa spp., were used as antigens. The antigen to be tested was prepared at 1:10 dilution (w/v) with coating buffer. From this, further antigen dilutions (v/v) were made at 1:50, 1:100, and 1:500, 1:1000, 1:5000 and 1:10,000 and tested by DAC-ELISA using rCP antiserum (1:5000, w/v). The mean A\textsubscript{405} values of DAC-ELISA are given in the table 6.2. A sample was considered to be positive if A\textsubscript{405} value is twice greater than the mean of the healthy controls (Rodoni et al., 1999). The values more than twice higher than the healthy controls were observed up to 1:500 antigen dilution (Table 6.2). The mean A\textsubscript{405} values of DAS-ELISA with 1:500 antigen and 1:10,000 antiserum dilutions were found to be five times greater than the mean values of the healthy control to detect BBrMV P1 (Philippine) isolate (Rodoni et al., 1999). Direct ELISAs like double antibody sandwich (DAS)-ELISA, simplified rapid direct antigen coating (SRDAC)-ELISA and indirect form of DAC-ELISA were used for detection of CMV causing infectious chlorosis of banana and found that the sensitivity levels of these three tests are similar (Kiranmai et al., 1996).

Table 6.2: Detection of BBrMV in Musa spp., leaf extracts by DAC-ELISA using polyclonal antibodies produced to rCP.

<table>
<thead>
<tr>
<th>Nature of antigen</th>
<th>1/10</th>
<th>1/50</th>
<th>1/100</th>
<th>1/500</th>
<th>1/1000</th>
<th>1/5000</th>
<th>1/10,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Control</td>
<td>0.43</td>
<td>0.31</td>
<td>0.27</td>
<td>0.24</td>
<td>0.21</td>
<td>0.16</td>
<td>0.11</td>
</tr>
<tr>
<td>Infected</td>
<td>1.28</td>
<td>0.83</td>
<td>0.76</td>
<td>0.62</td>
<td>0.40</td>
<td>0.29</td>
<td>0.20</td>
</tr>
</tbody>
</table>

* The values represent mean A\textsubscript{405} readings of two wells.
**6.2.2 Electro blot immunoassay**

Electro blot immunoassay is one of the widely used confirmative tests in the field of viral biology. In the present study, this experiment was carried out to detect BBrMV in *Musa* spp., leaf samples by using the homologous antiserum raised against its rCP. The healthy and infected leaf extracts were subjected to 12% SDS-PAGE and blotted onto the nitrocellulose membrane. The membrane was probed with fourth bleed antiserum of BBrMV and goat anti-rabbit antibodies labeled with ALP as primary and secondary antibodies at 1:10,000 and 1:2000 dilutions, respectively. Finally, the membrane was developed by incubating it in the substrate NCIP/NBT (Fig 6.1). The produced antibodies reacted with infected leaf extracts (1:10, w/v), but not with healthy leaf extracts, indicating the specificity of the antibodies. A band corresponding to the 38 kDa protein from partially purified leaf extracts reacted with potyvirus specific polyclonal antiserum in a western blot (Bateson et al., 1995). Antiserum to rCP of BBrMV (isolate P1) reacted strongly with 40 kDa protein present in a partially purified BBrMV preparation (Rodoni et al., 1999). This test is probably suitable for screening of *Musa* spp., samples on a small scale. It is sensitive but takes longer time to analyze the samples and may be more expensive compared to ELISA. It can be applied for BBrMV detection in *Musa* spp., on a small scale like screening of suckers in quarantine and in the selection of virus-free mother plants for micropropagation.

![Detection of BBrMV in Musa spp by electroblot immunoassay using homologous antiserum. Lane 1-healthy leaf extract, lane 2 and 3- infected leaf extracts.](image)

Fig 6.1 Detection of BBrMV in *Musa* spp by electroblot immunoassay using homologous antiserum. Lane 1-healthy leaf extract, lane 2 and 3- infected leaf extracts.
6.2.3 RT-PCR

RT-PCR is a novel technique widely used to detect RNA viruses in plant virology (Webster et al., 2004, Rao and Singh et al., 2008). In the current study, the technique was used to detect BBMV in Musa spp. Different RNA concentrations were made and used to detect BBMV by RT-PCR with specific primers to CP gene of the virus (Table 3.2). The total RNA was made into dilutions viz., 1:10, 1: 50, 1:100, 1:500, 1:1000, 1:5000 and 1:10,000 (v/v) and used as source of templates for RT-PCR amplification. After completion of experiment, the resulted product was resolved in 1% agarose and the amplification was observed up to 1:5000 dilution (Fig 6.2). It is clear that on equal weight basis of tissues this technique is nearly ten times more sensitive as compared to DAC-ELISA (Table 6.3).

![Fig 6.2 Detection of BBMV in Musa spp by RT-PCR](image)

It is a fact to say that the efficiency of RT-PCR in virus detection depends on successful RNA preparations. Sometimes the virus detection would fail by RT-PCR due to RNA degradation. For example, attempts to detect SSMV by RT-PCR has been hampered by technical difficulties in obtaining suitable RNA preparations from sugarcane tissue and the method is not amenable for large scale indexing. To mitigate the above difficulties, IC-RT-PCR was developed and used for detection of SSMV in sugarcane plants successfully (Hema et al., 2003).
6.2.4 IC-RT-PCR

An attempt was made to develop IC-RT-PCR to detect BBrMV in *Musa* spp. In the present study, the PCR vials were coated with PAbs to rCP at 1:500 dilution (v/v) and later the vials were loaded with leaf extracts of *Musa* spp., as antigen. Initially the antigen was prepared at 1:10 dilution (w/v) and later the same was used to prepare further dilutions (v/v) like 1:50, 1:100, 1:500, 1:1000, 1:5000 and 1:10,000. During the overnight incubation at 4°C the virions were trapped by the coated antibodies. After washing the vials were incubated at high temperature (65°C for 10 min.) to release the genomic RNA from the trapped virions. The released RNA was converted into first strand cDNA by using reverse transcriptase and the resulted cDNA used as a template for further PCR amplification. Amplification of CP gene of BBrMV with sequence specific primers was observed upto 1:10,000 antigen dilution, indicating its higher sensitivity over RT-PCR (Fig 6.3). It indicates that IC-RT-PCR successfully detected the virus in infected *Musa* spp. But the same technique failed to detect BBrMV PI isolate at higher dilutions of *Musa* spp. extracts, this could be due to RNA degradation during the overnight incubation (Rodoni et al., 1999). Harper et al (2004) used polyclonal antibodies raised against purified BSV to detect infectious episomal forms of virus in *Musa* spp., by IC-PCR. Polyclonal antibodies to CMV were used in multiplex-immunocapture-RT-PCR (M-IC-RT-PCR) for CMV detection in various banana cultivars collected from different countries (Sharman et al., 2000).

![Image](Fig 6.3 Detection of BBrMV in *Musa* spp. by IC-RT-PCR. Lane 1-DNA marker, lane 2 to 8 amplified IC-RT-PCR products of infected *Musa* spp. and lane 9-healthy sample)
6.2.5 Comparison of the sensitivity of DAC-ELISA, RT-PCR and IC-RT-PCR.

To know the sensitivity levels of the techniques used for the detection of BBrMV in *Musa* spp., the detection levels at different antigen/RNA concentrations are compared (Table 6.3). Initially, the antigen was prepared at 1:10 dilution (w/v) and from this further dilutions (v/v) 1:50, 1:100, 1:500, 1:1000, 1:5000 and 1:10,000 were made. For cDNA synthesis, total RNA from leaf tissues was also extracted at 1:10 proportion (w/v). The isolated total RNA was further diluted like antigen dilutions in a RNase free sterile distilled water and carried first strand cDNA synthesis. All these antigens and cDNAs dilutions were detected by antibodies or by genome specific primers, respectively. DAC-ELISA gave positive results upto 1:500 antigen dilution where as RT-PCR and IC-RT-PCR amplified cDNA upto 1:5000 and 1:10,000 dilutions, respectively (Table-6.3, Fig.6.2 and 6.3). It indicates that IC-RT-PCR is 20 and 2 times more sensitive as compared to ELISA and RT-PCR, respectively in detection of BBrMV in *Musa* spp.

It has been reported that IC-RT-PCR is more sensitive than the other antibody based techniques like ELISA, dot-ELISA and electro blot immunoassay (Rao and Singh et al., 2008). For example, the sensitivity of IC-RT-PCR is high as compared to ELISA and dot-ELISA employed to detect SS1MV in sugarcane (Hema et al., 2003). However, poor sensitivity of this test was reported in detection of BBrMV P1 isolate in *Musa* spp. This is due to the degradation of the BBrMV genome or due to the activity of endogenous inhibitors from banana sap during the overnight incubation step at 4°C which may prevent efficient reverse transcription of BBrMV genome (Rodoni et al.1999).

<table>
<thead>
<tr>
<th>Test</th>
<th>Antigen/cDNA dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAC-ELISA</td>
<td>+</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>+</td>
</tr>
<tr>
<td>IC-RT-PCR</td>
<td>+</td>
</tr>
</tbody>
</table>

+ : positive;  - : negative
6.2.6 Multiplex - PCR

Detection of individual viruses separately by RT-PCR or IC-RT-PCR reactions is expensive and time-consuming. Recently the technique multiplex-PCR has gained momentum in plant virology (Rao and Singh, 2008). Because, more than two viruses can be detected paralally in a single PCR vial by using this technique. Multiplex-PCR was developed for detection of several plant viruses. For example, multiplex-RT-PCR has been developed to detect five potato viruses viz., PVA, PVS, PVX, PVY and PLRV simultaneously in a single PCR tube (Nie and Singh, 2000). Similarly, a duplex-RT-PCR has been reported for detection of PLRV and PVY (Singh et al., 2000). In the present study a multiplex-PCR was developed to detect BBrMV, BSV and CMV infecting Musa spp. The selected genome regions like CP (389 bp), RT/ RNaseH (597 bp) and MP (830 bp) of BBrMV, BSV and CMV, respectively were amplified by this technique. Care was taken to design primers for their amplification. For BBrMV and CMV amplilication, total RNA was isolated by Qiagen RNaseasy isolation kit and synthesized first strand cDNA simultaneously in a single PCR vial by adding Oligo- dT and sequence specific reverse primer, respectively. To this cDNA reaction mix, total DNA isolated from BSV suspected Musa spp, was added and carried out the experiment as given in the protocol 3.2.26.3. The expected genome products for three viruses were amplified (Fig 6.4). The success of the technique also depends on the concentrations of the template. For example, during the optimization of the technique, it was found that addition of more genomic DNA template hampered the amplification of gene product of BBrMV. A multiplex-immunocapture PCR (M-IC-PCR) was developed for the simultaneous detection of three viruses viz., BBTV, BBrMV and CMV from crude sap extracts of banana and plantain (Musa spp.) in Australia (Sharman et al., 2000). Bariana et al (1994) have developed for simultaneous detection of seed -borne legume viruses like AMV, MYMV, CYVV, CMV and SCMoV that are great concern to legume germplasm banks. Thus the M-PCR developed in the present study could be useful for parallel detection of three viruses that cause similar symptoms at certain stages of disease development in Musa species. This
test can be used for screening of germplasm of *Musa* species and for selection of mother plants for mass multiplication by tissue culture technology.

Fig 6.4 Parallel detection of BBrMV, CMV and BSV in *Musa* spp., by multiplex-PCR. Lane 1- DNA marker, lane 2- BBrMV, lane 3- BSV, lane 4- CMV and lane 5- BBrMV (389 bp), BSV (597 bp) and CMV (830 bp).