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Virus-Indexing Technology for Production of Quality Banana Planting Material: a Boon to the Tissue-Culture Industry and Banana Growers in India

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Keywords: *Banana bract mosaic virus* (BBrMV), *Banana bunchy top virus* (BBTV), banana streak viruses (BSV), *Cucumber mosaic virus* (CMV)

Abstract

Banana and plantain (*Musa* spp.) are the most important fruit crop in India and play a major role in the livelihood of millions of resource-poor small farmers. Use of quality planting material is very important for increasing productivity. Though conventional suckers are still the primary planting material, use of tissue-culture plants has increased because of their advantages, like more uniform bunches with even maturity and increased yield. However, banana viral pathogens, which are economically important in India, can be inadvertently spread through tissue-culture plants. In order to control the spread of the viruses, virus-indexing techniques were developed at the National Research Centre for Banana, Tiruchirapalli for early detection in mother plants and tissue-culture plants used for mass propagation. Polymerase chain reaction (PCR), reverse transcriptase PCR (RT-PCR), non-radioactive probe-based nucleic acid spot hybridisation (NASH) and enzyme-linked immune-sorbent assay (ELISA) based techniques were developed and validated for routine testing. PCR and NASH tests are being done for detection of *Banana bunchy top virus* (BBTV) and *Banana streak Mysore virus* (BSMysV). RT-PCR and ELISA tests are being done for the detection of *Banana bract mosaic virus* (BBrMV) and *Cucumber mosaic virus* (CMV). Based on the work done at the centre, the Department of Biotechnology (DBT), Government of India, has accredited the Molecular Virology lab for testing for banana viruses in the country. In total, 15,850 tissue-culture and mother-plant samples were tested against viruses. The percentage of positive plants for BBTV, BSMysV, CMV and BBrMV were 3.83, 2.85, 17.3 and 0.95% respectively. Indexing was done mostly for the cultivars 'Grande Naine' (AAA, Cavendish subgroup), 'Robusta' (AAA, Cavendish subgroup) and 'Hill Banana' (syn. 'Virupakshi', AAB, Pome subgroup). Around 25 tissue-culture banana commercial laboratories are undertaking the services of virus testing. Our laboratory has also developed polyclonal antisera for CMV and BBrMV through recombinant DNA technology, which is currently being validated for ELISA-based testing.

INTRODUCTION

Banana is one of the most important staple food and fruit crops in the world. In India, it ranks first in production with 19.1 million tonnes produced annually from an area of 0.69 million ha. There are four important viral diseases affecting banana in India. *Banana bunchy top virus* (BBTV) of the genus *Babuvirus* in the family *Nanoviridae* is considered to cause the most serious viral disease of bananas worldwide (Dale, 1987). In India, it has almost wiped out the famous 'Hill Banana' (syn. 'Virupakshi', AAB, Pome), causing huge economic loss to the growers. It spreads primarily through infected planting materials, such as corms, bits or suckers or tissue culture-propagated plantlets and secondarily through banana black aphid, *Pentalonia nigronervosa*. *Banana streak Mysore virus* (BSMysV), a plant pararetrovirus, causes banana streak disease in 'Poovan' (AAB, Mysore). This important, widely-grown cultivar is almost 100% infected. BSV particles are non-enveloped, bacilliform shaped, 130-150×30 nm sized with a double- stranded

DNA as genome (Lockhart, 1986). The virus belongs to the genus *Badnavirus* in the family *Caulimoviridae*. BSV was reported in 1996 to occur in India (Selvarajan et al., 1997; Singh, 2003). *Banana bract mosaic virus* (BBrMV) is a distinct member of the genus *Potyvirus* in the family *Potyviridae*. It was first noticed in 1966 in cultivar 'Nendran' (AAB, plantain) from Kerala but the causal agent was not known then and the disease was described as Kokkan. Association of BBrMV with Kokkan in Nendran was first reported in 1996. Rodoni et al. (1997) reported BBrMV in Coimbatore and Tiruchirapalli districts of Tamil Nadu. The virus is transmitted by aphids (*Rhopalosiphum maidis*, *Aphis gossypii*, *A. craccivora* and *Pentalonia nigronervosa*) in a non-persistent manner (Magnaye and Espino, 1990 ; Selvarajan et al., 2006a). *Cucumber mosaic virus* (CMV) causes mosaic or infectious chlorosis disease. This virus belongs to the genus *Cucumovirus* in the family *Bromoviridae*. It was reported in the early 1940s in Maharashtra and later from many parts of the country (Kamat and Patel, 1951; Rao, 1980). The virus is transmitted through several aphid vectors in a non-persistent manner.

Viral pathogens are also a major impediment in the banana tissue-culture industry. They cannot be eliminated through shoot-tip culture which is the technique adopted by the industries for mass propagation. Most of the banana viruses reside in the host in latent form, i.e. without exhibiting any visual symptoms in the host for some period (sometimes for up to 2 years). BBTV-infected plants multiplied through tissue culture can be symptomless for over some months after planting out (Drew et al., 1989). Symptomless strains of CMV have been reported (Stover, 1972). In addition, symptoms of viral diseases often get confused with nutrient deficiency. CMV and BSV infection are often confused due to induction of similar symptoms. In order to make proper management decisions, early and correct diagnosis of viruses is important. This will help in checking further spread of pathogens, especially to a new area. Early detection by means of sensitive diagnostic methods is the main way to control them. If non-indexed plants are used as mother plants, viruses are spread, with huge effect on yield. Tissue culture is a widely adopted technology in horticultural industry. In order to sustain and reap the benefits of tissue-culture technology in banana, we have to ensure the growers have access to quality mother plants that have undergone virus indexing for producing healthy and high-yielding planting material. Different molecular diagnostic techniques that are used worldwide by the tissue-culture industry to detect banana viruses in tissue-culture plants and mother plants used are reviewed in this paper. Diagnostic techniques for banana viral pathogens developed by National Research Centre for Banana (NRCB) are also discussed.

INDEXING TECHNIQUES FOR BANANA VIRUSES

Serological Detection Techniques

Various forms of serological assays are currently available for all seven known banana viruses. Enzyme Linked Immuno Sorbent Assay (ELISA) tests with monoclonal antibodies (Mabs) have been commonly used for the accurate detection of BBTV (Wu and Su, 1990; Thomas and Dietzgen, 1991; Geering and Thomas, 1996; Espino et al., 1989). Geering and Thomas (1996) developed triple antibody sandwich (TAS)-ELISA for routine virus indexing of BBTV. Wu and Su (1990) produced monoclonal antibodies and detected BBTV by plate-trapped antigen (PTA)-ELISA. Wanitchakorn et al. (1997) developed recombinants expressing BBTV coat protein and raised polyclonal antiserum which satisfactorily detected the virus in asymptomatic plants. Selvarajan et al. (2002) produced polyclonal antiserum for BBTV 'Hill Banana' isolate and reported that the direct antigen coating (DAC)-ELISA method was more sensitive than dot immuno binding assay (DIBA) for detection of BBTV. BBrMV has been detected by serology using ELISA (Espino et al., 1990; Singh et al., 2000; Thomas et al., 1997; Selvarajan et al., 2006b). Espino et al. (1989) developed monoclonal antibodies for BBrMV and detected the virus by double antibody sandwich (DAS)-ELISA. The coat protein of BBrMV was expressed in *Escherichia coli* as a fusion recombinant protein and was used

to produce a high-titre BBrMV-specific polyclonal antiserum for serological assays (Rodoni et al., 1997). Detection of BSV has been problematic due to serological and genomic heterogeneity of virus isolates (Lockhart and Olszewski, 1993). Thottappilly et al. (1998) produced high-titre polyclonal antibodies against Nigerian isolates of BSV. They reported that TAS-ELISA was more sensitive than antigen-coated plate (ACP)-ELISA and protein-A coated antibody sandwich trapped (PAS)-ELISA. Agindotan et al. (2003) reported high-titred monoclonal antibodies for BSV, which can detect all the isolates of BSV. Agindotan et al. (2006) reported that IC-PCR was considerably more sensitive than immunoelectron microscopy (IEM) for detecting typical BSV, while IEM proved to be of similar sensitivity as TAS-ELISA by sap dilution end-point analyses. Kiranmai et al. (1996) have demonstrated potential applicability of DAC-ELISA in large-scale indexing of banana for CMV infection.

Nucleic-Acid or Viral Genome-Based Methods: Nucleic Acid Spot Hybridisation

We have developed Nucleic Acid Spot Hybridisation (NASH) technique for detection of BBTV in tissue-culture samples which is equally sensitive as Polymerase Chain reaction (PCR). The same technique has been applied for detection of BBrMV, CMV and BSMysV (R. Selvarajan, unpublished). Xie and Su (1995) used 32P and digoxigenin (DIG)-labelled probes for detection of BBTV in Australia. The integrated BSV genome in the *Musa* genome that can cause episomal infection under certain circumstances was detected by southern analysis (Harper et al., 1999; Ndowora et al., 1999). BBrMV detection using DIG-labelled virus-specific probes was more sensitive than ELISA. BBrMV was detected by specific DIG-labelled non-radioactive probes (Rodoni et al., 1997). Bateson and Dale (1995) developed northern blot assay using radioactive labelled probes to detect RNA from BBrMV-infected banana tissue. The virus can be detected by non-radioactive nucleic acid probe specific to BBrMV (Thomas et al., 1997). Kiranmai et al. (1998) used dot blot hybridisation technique to detect CMV-banana isolate with 32P-labelled radioactive probe as well as DIG-labelled probes. Heterologous 32P-labelled probe prepared for CMV infecting pepper successfully detected the CMV-banana isolate (Srivastava et al., 1995). DIG-labelled non-radioactive DNA probe has been used to detect CMV in sap extracted with pinpricking the pseudostem. This method is simpler and less expensive than routine time-consuming preparation of extracts (Kiranmai et al., 1998).

Polymerase Chain Reaction (PCR)

PCR-based detection systems are now also available for all banana viruses (Dietzgen et al., 1999; Harper et al., 1999). Xie and Hu (1995) used PCR for detecting the Hawaiian isolates of BBTV, and it was 1000 times more sensitive than ELISA or dot blots with DNA probe. A simple, single-step plant-tissue preparation protocol to reduce plant inhibitory factors interfering with PCR suitable for the detection of BBTV in corm, leaf and root tissues by PCR was developed (Thomson and Dietzgen, 1995). Mansoor et al. (2005) detected a Pakistan isolate of BBTV by PCR and used primers for banana genomic sequences as an internal control for overcoming the uncertainty over inherent PCR. Selvarajan et al. (2007) also developed a PCR-based detection method for Indian isolates of BBTV. PCR has been used to detect BBTV from viruliferous aphids (Manickam et al., 2002; Selvarajan et al., 2006b). BBrMV was detected by RT-PCR in total nucleic acid extracts from infected plants, using specific or degenerate potyvirus group primers (Bateson and Dale, 1995; Thomas et al., 1997). Indian isolates of BBrMV were detected from pseudostem and banana bracts through reverse transcription (RT)-PCR (Sankaralinkam et al., 2006; Selvarajan et al., 2006b). A Kerala isolate of BSV was detected by PCR using primers specific to conserved domains of RT/RNaseH region of the genome of badnavirus (Cherian et al., 2004). Singh et al. (1995) and Hu et al. (1995) used RT-PCR reaction assay for detection of CMV infecting banana.

BANANA VIRUS INDEXING IN INDIA

In India, tissue-culture banana plants are produced on a large scale, involving nearly 25 firms. In order to ensure quality tissue-culture plants from the industries, the Department of Biotechnology (DBT), Government of India, along with the Indian Council of Agricultural Research (ICAR), has developed specific standards for banana tissue-culture industries for certification. The steps involved in the production of tissue-culture banana include selecting good mother plants with no visible symptoms of virus diseases, and virus indexing to confirm that the plants are free of any latent infection of four of the known viruses. Indexing has to be done at random during subculturing and hardening stages. DBT has accredited labs for virus testing for various crops, such as apple, citrus, banana, vanilla, pepper, potato and sugarcane, which are mass propagated by tissue culture in India. For banana, the Molecular Plant Virology Lab of NRCB, Trichy has been accredited to take up virus indexing for banana tissue-culture plants produced in India. At NRCB, BBTV has been purified from infected 'Hill Banana' plants and raised polyclonal antiserum which had a titre of 1:250. Serodiagnostic techniques, like DAC-ELISA and DIBA, were standardised and used for the detection of BBTV. Serodiagnostic techniques were also standardised for BBrMV and CMV. Recently, we have raised polyclonal antiserum for the recombinant coat proteins of BBrMV and CMV (R. Selvarajan, unpublished). For each virus, specific primer pairs were designed, tested and validated for PCR-based detection. In case of RNA viruses, such as BBrMV and CMV, primers for coat protein genes were targeted and RT-PCR technique with high sensitivity was developed and validated for use in virus indexing. For handling a higher number of samples at a time, a non-radioactive probe-based NASH technique has been standardised and validated for detecting all four banana viruses.

Since 2004, virus-testing work is being taken up for private farms, horticultural departments and for farmers. Details of year-wise samples tested for each virus are furnished in Table 2. In total, 15,850 tissue-culture and mother-plant samples were tested against viruses. The percentage of positive plants for BBTV, BSMysV, CMV and BBrMV were 3.83, 2.85, 17.3 and 0.95% respectively. Though many techniques were often employed for detection of banana viruses, PCR was the best for detection of BBTV than serological techniques. BBrMV and CMV are best to be indexed by RT-PCR. PCR and IC-PCR are suitable for the detection of BSMysV. Indexing was done mostly for the cultivars 'Grande Naine' (AAA, Cavendish subgroup), 'Robusta' (AAA, Cavendish subgroup) and 'Hill Banana' (syn. 'Virupakshi', AAB, Pome). The test results showed that CMV and BBTV were present in more samples than BSMysV and BBrMV. BBTV, BSMysV and CMV have been detected from samples received from both south and western parts of India whereas BBrMV was mostly from south India. Testing was also done for germplasm samples received from different states for timely elimination of virus-infected plants.

CONCLUSIONS

A wide range of molecular diagnostic techniques are available for many plant viruses. However, it is important to develop cost-effective, easy-to-use, reliable and sensitive techniques for routine virus indexing. Though the antiserum for BBTV is available commercially, its sensitivity needs to be compared with nucleic acid-based techniques. Recently, many species of BSV are being reported. Though a cocktail of antiserum has been used to detect episomal viruses through immunocapture (IC)-PCR technique, some of the new variants observed under electron microscope could not be detected (Agindotan et al., 2006). It shows that further characterisation of many isolates in different banana-growing regions of the world has to be done and, as a consequence of the variability, a good technique has to be worked out for reliable detection of all the types of variants of BSV. To reduce the cost of indexing for tissue-culture plants, it is necessary to develop multiplex PCR for the simultaneous detection of all four banana viruses. The growing need to index for banana viruses in India requires more attention to the design and development of new techniques, like microarray, immunostrip and

multiplex PCR. Nanotechnology might also play a role in diagnosis of plant viruses for improving the sensitivity of detection in coming years.

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Tables

Table 1. Indexing techniques being used for banana virus diagnosis at NRCB, India.

List of viruses	Test method developed and used at the lab			
	PCR / RT-PCR/ IC-PCR/ DB-PCR/ IC-RT-PCR	ELISA /DIBA	Nucleic acid spot hybridisation (NASH) using non-radioactive DNA / RNA probes	Southern and northern blots using Non-radioactive probes
BBTV	√	√	√	√
BBrMV	√	-	√	√
BSMysV	√	-	√	√
CMV	√	√	√	√

Table 2. Details of year-wise samples tested for banana viruses.

Year	No of samples tested	No of positives			
		BBTV	BSMysV	CMV	BBrMV
2004-05	1072	-	21	7	-
2005-06	3192	126	20	-	-
2006-07	1102	15	4	-	-
2007-08	3623	38	2	77	-
2008-09	6681	428	404	85	15