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# PCR Detection of Banana Bunchy Top Virus (BBTV) at Tissue Culture Level for the Production of Virus-free Planting Materials

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## Abstract

*Banana bunchy top disease, caused by Banana bunchy top virus (BBTV), is a serious disease that affects the productivity of banana to a greater extent. The production of virus-free planting material is an efficient method in controlling this disease and increase productivity. In vitro propagation, especially meristem tip culture, has played a key role for obtaining a large number of virus free, homogenous planting materials in plantains and bananas (Musa spp.). In our previous study we have demonstrated that direct regeneration from male floral meristems is a rapid and simple method for clonal and mass propagation of Musa spp. cultivars 'Virupakshi' and 'Sirumalai' (AAB). Detection of BBTV by polymerase chain reaction (PCR) is very reliable and economically important for testing micropropagated banana plantlets for the absence of virus before release to farmers. In this study we have developed a protocol for early diagnosis of BBTV at tissue culture level itself through optimizing the Polymerase Chain Reaction (PCR) method. We designed the viral gene specific primers [coat protein (CP) gene and nuclear shuttle protein (NSP) gene] and standardize PCR conditions for detection of presence of BBTV. All the regenerated plantlets were tested for BBTV presence with standardized PCR and found to be completely free of virus. PCR tests are more sensitive than ELISA tests and are better to be suited to plant tissue with lower number of pathogens, such as in tissue culture conditions.*

**Keywords:** Banana bunchy top disease, polymerase chain reaction, banana bunchy top virus, nuclear shuttle protein gene, coat protein gene, gene specific primers.

## Introduction

Banana (*Musa* spp.) is worldwide an important food and cash crop. Banana cultivation constitutes an important socio-economical activity in around 80 countries, mainly on small farms. Banana bunchy top disease (BBTD), caused by Banana bunchy top virus, was first discovered in the Fiji islands in 1889 and spread in many parts of the world, including Asia, Africa, Australia and South Pacific. It has become the most economically important viral disease of banana[1]. The transmission vector for this disease is the banana aphid (*Pentalonia nigronervosa*) following a minimum acquisition-access period of approximately 4 hours and a minimum inoculation-access period of about 15 min and is then retained for the life of the aphid vector[2]. This virus also spread through infected planting material like plant suckers and other plant tissues used in banana propagation.

BBTV (genus *Babuvirus*, family *Nanoviridae*) is an isometric virus with a circular single stranded DNA (ssDNA) genome consisting of at least six components (BBTV DNA-1 to 6). Each DNA component has two conserved regions, the stem-loop common region (CR-SL) and the major common region (CR-M)[3]. Previous studies[4,5,6] have shown that components 1, 3, 4, 5 and 6 encode the replication-associated protein, coat protein, intercellular transport protein, retinoblastoma binding protein and nuclear shuttle protein, respectively. No putative function has been identified for BBTV DNA-2.

Use of free BBTV-free planting material coupled with regular rouging (removal and destruction) of infected plants constitute the best ways to control BBTD[7]. Banana regeneration has been achieved from various explants including suckers, shoot tips, zygotic embryos, immature male flowers, multiple meristem and anthers[8-12]. Considering the genotypic influence on *in vitro* growth, specific protocols are essential for different banana cultivars. In addition, Enzyme-linked immunosorbent assay (ELISA) tests with monoclonal antibodies are commonly used for the accurate detection of BBTV[13-15]. ELISA is convenient but use of this technique is limited in detection sensitivity with very low concentrations of BBTV. Therefore, a more sensitive assay based on PCR has been developed for indexing mother plants or for certifying propagules[16-18].

In this study, we standardized the PCR protocol for early diagnosis of BBTV for virus-free planting materials of *Musa* spp. cultivars 'Virupakshi' and 'Sirumalai' (AAB) even at tissue culture level itself where the virus load is very low. Viral gene

specific primers (CP and NSP gene) were synthesized according to the conserved sequences in BBTV DNA-3 and DNA-6 respectively; these conserved regions occur in almost all published sequences. The standardized PCR protocol was successfully applied for the early detection of the BBTV in regenerated plantlets of *Musa* spp. cultivars 'Virupakshi' and 'Sirumalai' (AAB).

## Material and Methods

**Regeneration of hill banana plantlets:** The work was carried out in the Department of Plant Molecular Biology and Biotechnology, Centre for Plant Molecular Biology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. Floral apices of *Musa* spp. cv. 'Virupakshi' and 'Sirumalai' (2n=3X=33, AAB) were collected from farmers' fields at the lower Pulney hills region, Tamil Nadu, India. The plantlets were regenerated using protocol as per previously described protocol[12].

**Total genomic DNA extraction from plant tissues:** The fresh green leaves collected from mother plants while collecting explants and green portion (figure-1) collected during sub-culturing in tissue culture were used for DNA extraction. The samples were stored at –70 °C for further use.

The total DNA was isolated using the modified CTAB protocol. Prior to extraction, 100 to 300 mg of collected samples were cut in to bits and transferred to a Ziploc bags (7 x 9 cm). The bag was immediately placed in liquid nitrogen for 1 min, and immediately 500 µl of extraction buffer [Tris–HCl-10 ml of 1M (pH.8.0), EDTA-10 ml of 0.5 M (pH.8.0), NaCl- 10 ml of 5 M, Sodium diethyldithio carbonate- 225 mg] was added. The samples were kept in room temperature and squeezed by rolling a glass rod over the sample to extract the cell contents. 500 µl of the cell extract was transferred in to an eppendorf tube, and then 33 µl of 10 % SDS was added into the tube and mixed well by vortexing. The tube was kept in water bath at 65 °C (pre-heat treatment) for 10-12 min. Then the tube was centrifuged for 10 min at 12,000 rpm and 450 µl of the supernatant was transferred immediately to a new eppendorf tube. The tube was stored in –20 °C after adding 0.7 volume of ice cold IPA (isopropyl alcohol) and then tube centrifuged for 15 min at 12,000 rpm. After centrifugation the supernatant was discarded without disturbing the pellet and 50 µl of 70 % ice cold distilled ethanol was added to the pellet and centrifuged for 5 min at 12,000 rpm. The supernatant was discarded and the pellet was air dried. The dried pellet was dissolved in 40 µl of 0.1X TE buffer and kept at 65 °C for 3 min (to suspend the pellet). The suspended DNA sample was stored at -20ºC. DNA.

**Electrophoreses:** Purity of the isolated DNA was checked with agarose (0.8 %) gel electrophoresis and quantified by UV/Vis spectrophotometer (Nanodrop Technologies, ND-1000, USA). Electrophoresis was carried out on agarose (0.8 % of w/v) gel at 80 Volt for 1 hour. The DNA bands were visualized under gel documentation system (Alpha Imager, Alpha Innotech Corp., CA, USA) and photographed.

**PCR assay:** Gene specific primers were designed to amplify the coat protein and nuclear shuttle protein genes of the virus (table-1). PCR amplification was performed for mother plants and tissue culture derived plants. Various parameters like concentration of template nucleic acid and $MgCl_2$ were optimized (data not presented). Reactions were performed in a final volume of 20 µl [2 µl of diluted total genomic DNA, 2.0 µl of 10X PCR buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 0.5 µl of 200 mM dNTPs, 0.5 µl of 70 ng of respective forward and reverse primers, 0.5 µl of 1.5 µl Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India) and 16.5 µl of sterilized double distilled water]. Amplification was performed in a PTC-100™ Programmable Thermal cycler (MJ Research, Inc., Watertown, USA).

**Table-1**
**Primers designed for virus indexing**

| Name | Sequence | Length (bp) | PCR Amplification product size (bp) |
|---|---|---|---|
| CP.F1 | 5' ATGGCTAGGTA TCCGAAG 3' | 18 | 530 |
| CP.R1 | 5' CCAGAACTACA ATAGAATGCC 3' | 21 | |
| NSP.F1 | 5' CCTCGCAAGGT ACTTCTTAG 3' | 20 | 237 |
| NSP.R1 | 5' CCATGTCTCTGC TCCAATCT 3' | 20 | |

CP.F1 and CP.R1- Coat protein gene specific forward and reverse primer; NSP.F1 and NSP.R1- Nuclear shuttle protein gene specific forward and reverse primer.

The temperature profile used in the PCR for CP gene was as: an initial denaturation for 5 min at 94ºC, then 35 cycles of: 1min at 94ºC, 1 min at 57ºC, 90s at 72ºC, and then a final extension for10 min at 72ºC. The temperature profile used in the PCR for NSP gene was as follows: an initial denaturation for 5 min at 94 ºC, then 35 cycles of: 1min at 94ºC, 1 min at 50.4ºC, 90s at 72ºC, and then a final extension for 10 min at 72ºC.

## Results and Discussion

**Total nucleic acid extraction:** BBTV has been the sole cause for reduction in hill banana cultivation from 18,000 ha in 1970s

to a mere 2,000 ha at present. Our previous study revealed that direct regeneration from virus-free male floral meristem is a rapid and simple method for clonal and mass propagation of *Musa* spp. cultivars 'Virupakshi' and 'Sirumalai' (AAB) were confirmed with PCR detection method, that can be applied on a commercial scale[12]. Total genomic DNA was extracted for BBTV detection from removable parts and leaf samples collected while sub-culturing (figure-2). Leaf samples of apparently infected plants were collected from different locations and total genomic DNA was isolated as positive control for standardization of PCR conditions.

**PCR detection analysis:** All DNA samples were tested for BBTV gene specific primers (CP and NSP gene) with PCR. Interestingly, all the tested regenerated plantlets were found to be negative and all the samples of apparently infected plants confirms the presence of BBTV with amplicon size of 530 bp and 237 bp (DNA-3 and DNA-6 respectively) (figure-3 and 4).

For virus-free plantlets production, the source plants for tissue culture must be completely free of virus. Essential component for the virus-free planting material production is the virus indexing technology. An effective management strategy is dependent on rapid detection of infected plants so that potential source plants of BBTV can be destroyed promptly. Early detection of BBTV, either in the symptomless young plants or in micropropagated planting materials plays an important role in its control[1,19,20].

Detection of BBTV by PCR was reported using primers specific to coding sequences of coat protein and replicase[21]. Most of these studies showed successful detection of symptomatic plant. Although tissue culture is believed to eliminate any risk that plantlets might be carrying fungal, bacterial or nematode pathogens or insect pests of banana, micropropagated plantlets could still be carrying virus pathogens if the source plants used for culture initiation are infected with virus. Here, we present data showing that amplification based on DNA-3 and DNA-6 sequence (CP and NSP gene respectively) was effective in detecting the presence of virus even at tissue culture level too. The problem of low titer of the virus in general and seasonal variation in the viral template concentration was overcome here. This particular screening was very important for tissue cultured plants as the industry always reported somaclonal variants in the generated plant populations that looked quite like bunchy top syndrome. Virus-free planting material production in banana could help in promoting the banana cultivation.

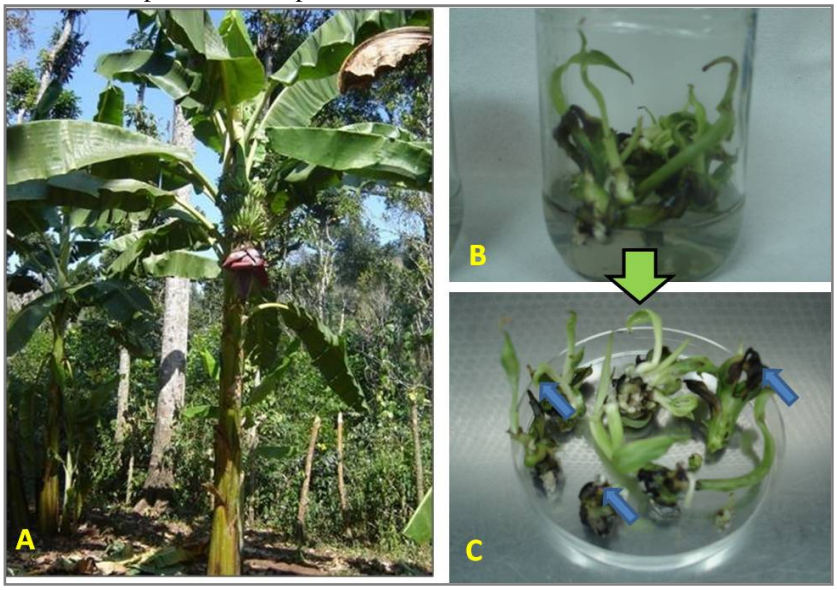


**Figure-1**
**Mother Plant (A), *Musa* spp., hill banana *cv.* Virupakshi or Sirumalai (AAB). B and C. Plant tissue (indicated with blue arrows) used for plant total DNA extraction from tissue culture method**

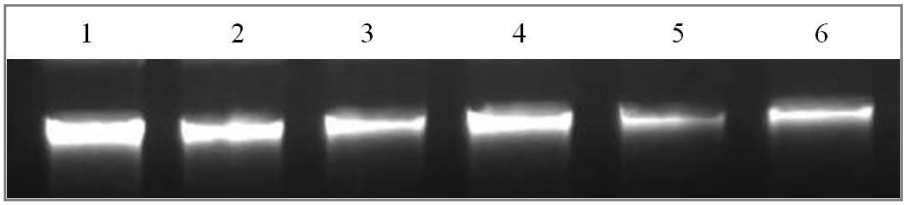


**Figure-2**
**Plant total DNA check for micropropagated shoots**

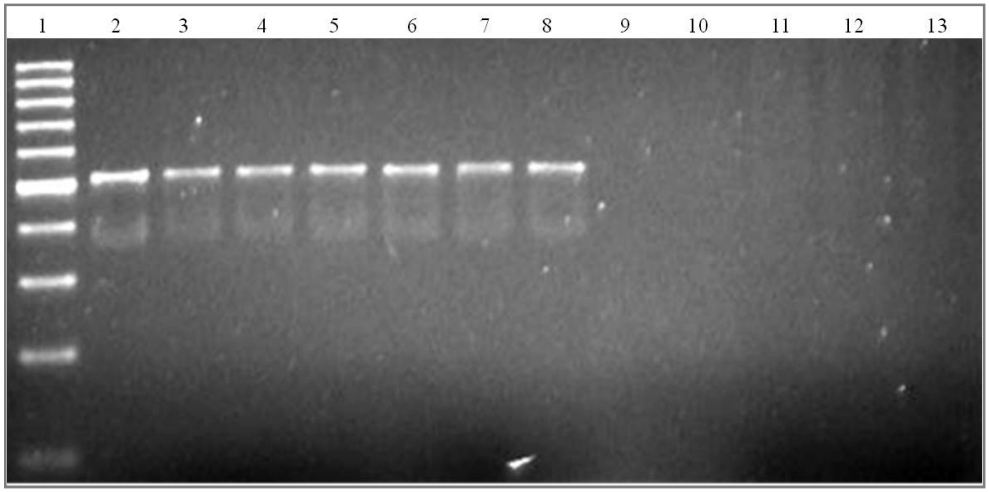


**Figure-3**
**Virus indexing with PCR using CP gene specific primers, Lane 1: 100 bp ladder; Lane 2, 3, 4, 5, 6 7 & 8: Positive control (infected samples collected from the different locations) Lane 9, 10, 11, 12 & 13: DNA from micropropagated shoots. PCR product amplification size is 530 bp**

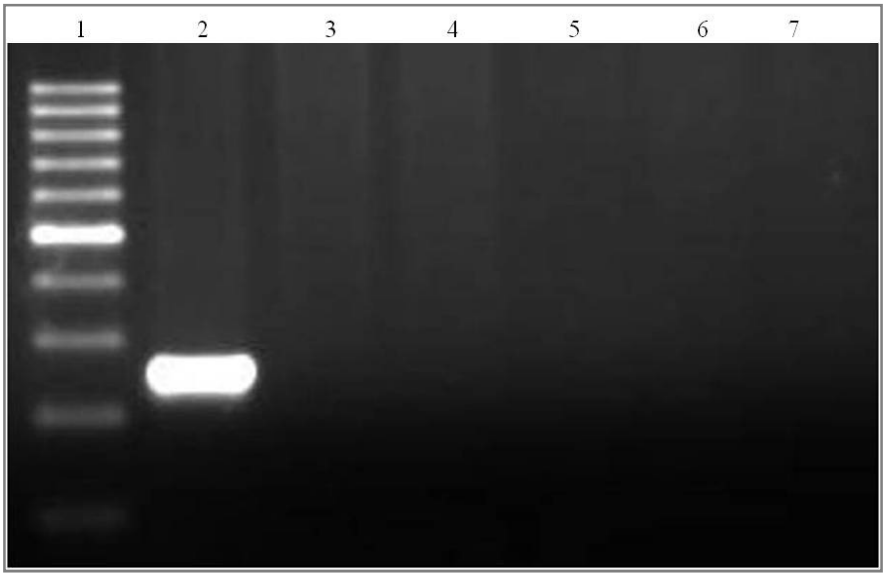


**Figure-4**
**Virus indexing with PCR using NSP gene specific primers, Lane 1: 100 bp marker; Lane 2: Positive control (Infected Samples collected from the different locations as positive control). Lane 3, 4, 5, 6 & 7: DNA from micropropagated shoots. PCR Product amplification size is 237 bp**

## Conclusion

The PCR technique was standardized and successfully applied for BBTV detection even at tissue culture level where virus load is very low. Devising an efficient method of diagnosing the infection of BBTV virus is very important and development of such protocol will be of great help to the banana growers. The described method is advantageous than alternative BBTV detection techniques such as enzyme-linked immunosorbent assay and more economical to reduce the cost for production of virus-free planting materials. In this context, PCR-based detection methods will have the advantage of amplifying the target nucleic acid present even at very low level and it is an attractive technique for the early diagnosis of plant viral diseases.

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