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A SUITABLE METHODOLOGY TO DETECT BANANA STREAK VIRUS (BSV) IN TISSUE CULTURED BANANA

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ABSTRACT

Banana is one of the most important fruit crops in Sri Lanka. Four viruses known to infect banana have been identified as Banana Bunchy Top Virus (BBTV), Banana Streak Virus (BSV), Banana Bract Mosaic Virus (BBrMV) and Cucumber Mosaic Virus (CMV). Among them BSV can rapidly spread through micro propagation. This is due to the integration of BSV genome in chromosomal DNA in some *Musa* cultivars having the B genome. During stress conditions, especially through the tissue culture process, integrated sequence gives rise to episomal infection of BSV. Therefore, development of a suitable method to identify BSV free tissue cultured banana plants is highly important. This integration made it difficult for diagnosis of BSV at DNA level and special precautions have to be made to distinguish integrated DNA and episomal viral DNA. Polymerase Chain Reaction (PCR) and Immuno Capture Polymerase Chain Reaction (IC-PCR) methodologies were compared to detect integrated form and episomal BSV in banana produced through tissue culture. Twelve different varieties having genomic configuration of A and B were tested for BSV using Mys F1 and Mys R1 primers. All the cultivars with B genome were positive for PCR and only plants which showed specific symptoms of BSV were positive for IC-PCR. Furthermore, PCR and IC-PCR results were compared with expressions of symptoms in field grown tissue cultured banana. The study revealed that IC-PCR methodology can be used to distinguish the episomal BSV and successfully applied for virus indexing during the tissue culture process in order to produce BSV free planting material.

KEYWORDS: Episomal viral DNA, Immuno capture polymerase chain reaction, Integrated DNA, Polymerase chain reaction.

INTRODUCTION

Banana (*Musa spp.*) is one of the most popular fruit crops with a year round production in Sri Lanka. It is grown both as a staple fruit and as a cash crop mainly for the local market. It is also an attractive perennial fruit crop for farmers due to its high economic gains throughout the year. Currently, banana cultivation in Sri Lanka is affected by four major viral diseases named as Banana Bunchy Top Virus (BBTV), Cucumber Mosaic Virus (CMV), Banana Streak Virus (BSV) and Banana Bract Mosaic Virus (BBrMV). Banana streak virus, the causal agent of viral leaf streak, is considered to be the most frequently occurring virus of *Musa* in the world. Presence of BSV in Sri Lanka was first confirmed by Thomas *et al.* in 1997. It is a member of Family Caulimoviridae, and Genus *Badnavirus*. Moreover, it contains a circular double stranded DNA genome of 7.4 kb in size (Lockhart and Olzewsk, 1993).

Chlorotic streaks and flecks are prominent symptoms in the leaf lamina of infected plants and these streaks become progressively darker and necrotic as the leaf ages eventually (Fig. 1). An important feature of the disease is that streak expression is periodic; plants may not show streaks on all leaves for months at a time and emerging leaves may not show symptoms (Singh, 2003).

According to Harper *et al.* (1999) all *Musa* spp examined so far harbour some form of BSV sequence in their DNA. Activation of the virus to cause episomal infection seems to be due to certain stresses on the plant and in the case of BSV one of the main activation triggers has been shown to be vegetative propagation of *Musa* material by tissue culture. Banana plants with B genes in their genome, with no episomal virus particles in the cells, undergo vegetative multiplication by tissue culture and produce progenies containing systemic episomal BSV. This phenomenon has never been observed with Cavendish cultivars which contain the A genome and are regularly multiplied by tissue culture (LaFleur *et al.*, 1996; Ndowora *et al.*, 1996; Harper *et al.*, 1999; Dallot *et al.*, 2001).

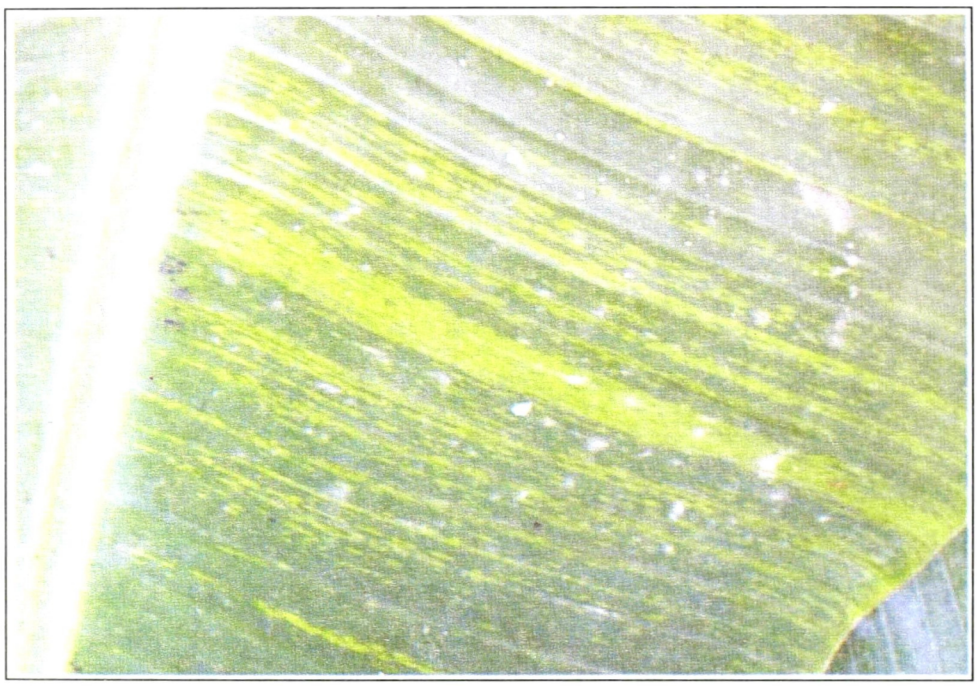

Figure 1. Chlorotic streaks in the leaf lamina of BSV infected banana plants.

As BSV endogenous sequences are found in the genome of most *Musa* species, it remains important to distinguish between episomal and integrated forms of BSV. Immuno Capture Polymerase Chain Reaction (IC-PCR) is a detection method in which actual viral particles are trapped with specific antibodies. This is a useful technique for determining the presence of Banana Streak Disease (BSD) caused by episomal BSV. IC-PCR is usually

performed in a single PCR tube in which antibodies are bound on the inner tube surface. However, several steps such as washing the tubes with a specific buffer between antiserum and sample incubation steps are needed before PCR is performed. These washing steps are employed in order to eliminate all the traces of plant material. BSV specific antibodies are also crucial in this procedure. Although antiserum addition and washing steps are employed, a slight chance always exists that traces of plant material may end up in the PCR reaction especially where large amount of samples are being processed. PCR is a fairly sensitive method and if primers recognized the sequence it can be multiplied (Old and Primrose, 1994).

It has been found that the local variety "Embul" (AAB), which is more susceptible to BSV, has the highest demand for cultivation and it is now being micropropagated widely (Gimhani *et al.*, 2005).

The objective of this study is to find the suitability of IC-PCR to detect Banana Streak Virus (BSV) in *in vitro* cultured banana plants with B genome to confirm the induced BSV infection.

MATERIALS AND METHODS

This study was conducted at the Plant Virus Indexing Centre (PVIC), Gabadawatta, Homagama from January 2005 to December 2007. The incidence of episomal BSV in tissue cultured banana varieties were determined by sampling at the *in vitro* stage in the tissue culture laboratory, hardening stage at the insect protected plant house and at the field. Tissue culture propagation was done using culture media of MS + BAP (Benzyl Amino Purine) 5 mg/l for shoot multiplication and MS + 0.1mg IAA (Indole Acitic Acid) for root growth. For screening, samples were selected from the planlets of the 6^{th} subculture at the *in vitro* stage, from the plants two weeks after introduction for hardening, and the tissue cultured plants grown six months in the field. Eight individuals were screened for each cultivar in three stages. Positive and negative controls for BSV were obtained from the plants grown in insect proof plant houses. Furthermore, samples with typical BSV symptoms confirmed by routine virus indexing programme were also used as positive controls.

Sample preparation and analysis

Extraction of total nucleic acid (Su's protocol, 1999)

A leaf lamina tissue of 0.5 g was ground into a fine powder using liquid nitrogen in a mortar and pestle. Then 2.7 ml of DNA extraction buffer (100 mM Tris HCl,100 mM EDTA, 250 mM NaCl at pH 8.0) and 0.3ml of 10% sarcosyl were immediately added to it. The powder suspension was stirred well and transferred to 1.5 ml eppendorf tubes and incubated at 55°C

for 1 h in a water bath. Tubes were centrifuged at 6000 rpm for 5 min. Then 800 µl of aqueous phase was transferred to a fresh tube and 100 µl of 5 M NaCl and 100 µl 10% Cetyl Trimethyl Ammonium Bromide (CTAB) in 0.7 M NaCl were added. After that tubes were incubated at 65°C for 10 min. To this, 500 µl of chloroform: isoamyl alcohol was added at 24:1, mixed thoroughly and spun at 11000 rpm for 5 min. To the saved aqueous suspension of 800 µl, 600 µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added, mixed thoroughly and centrifuged at 11000 rpm for 5 min. To the saved supernatant, 0.6 volume of isopropanol was added and incubated at -20°C for overnight. Then the tube was centrifuged at 12000 rpm for 15 min. The pellet was collected and washed with 70% ethanol to remove CTAB residuals, briefly dried and resuspended in 50 µl of TE buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA).

Polymerase Chain Reaction (PCR) amplification

The PCR mix consisted of 5 µl of 5X PCR buffer, 1.75 µl of 25mM $MgCl_2$, 200 mM of each dNTPs, 200 nmol of each of primers of Mys F1 and Mys R1, 1.25 units of Taq polymerase (Promega), 3 µl of DNA template and deionized water to give a total volume of 25 µl. The amplification conditions were initially 94°C for 30 sec, 35 cycles at 94°C for 10 sec, 64°C for 30 sec, 72°C for 1 min and finally 72°C for 5 min.

Immuno Capture Polymerase Chain Reaction (IC-PCR)

Thin walled 500 µl PCR tubes were coated with 50 µl of BSV polyclonal Ig (AGDIA) at 1:200 dilution in sterile 0.05 M sodium carbonate buffer (pH 9.6) and incubated for 3 h at room temperature. Then the tubes were washed 3 times for 3 min. with PBS-T buffer. To prepare leaf extracts for testing by IC-PCR, 0.2 g of leaf material was ground in a mortar and pestle with 1 ml of specific virus extraction buffer [13% Na_2SO_3, 0.02% NaN_3, 2% PVP, 2% tween-20, 0.2% albumin in Phosphate Buffered Saline (PBS) / 0.5 ml tween 20 (PBS-T)] The leaf extract was clarified by centrifuging at 6000 rpm for 5 min. and after centrifugation, 50 µl of the supernatant was added to the tubes and incubated overnight at 5°C. Then tubes were washed with 50 µl of PBS-T buffer for three times. Then the PCR mix, that was prepared as described above except template was added to the tubes and subjected to amplification conditions described above.

Detection of PCR and IC-PCR products

PCR and IC-PCR products (15 µl) were analyzed on 1% agarose gel in TAE buffer (0.04 M Tris acetate 0.001 M EDTA pH 8) and visualized by ethidium bromide staining under UV transilluminator and photographed using USB digital camera.

RESULTS AND DISCUSSION

In this study 12 banana cultivars, micropropagated through tissue culture at PVIC were screened. Geering *et al.* (2005) reported that at least part of the genome of BSV "Mysore strain" was integrated into banana B genome. In Sri Lanka PCR results were positive mostly for the BSV strain Mysore (Gimhani *et al.*, 2005). Therefore, in this study primer pair of Mys 1 and Mys 2 was selected for the screening of above cultivars. Mys F_1 and Mys R1 target the sites within rhibonulease H domain of the open reading frame 111 polyprotein in BSV genome (Geering *et al.*, 2000). This primer pair amplified the product size of 589 bp (Fig. 2).

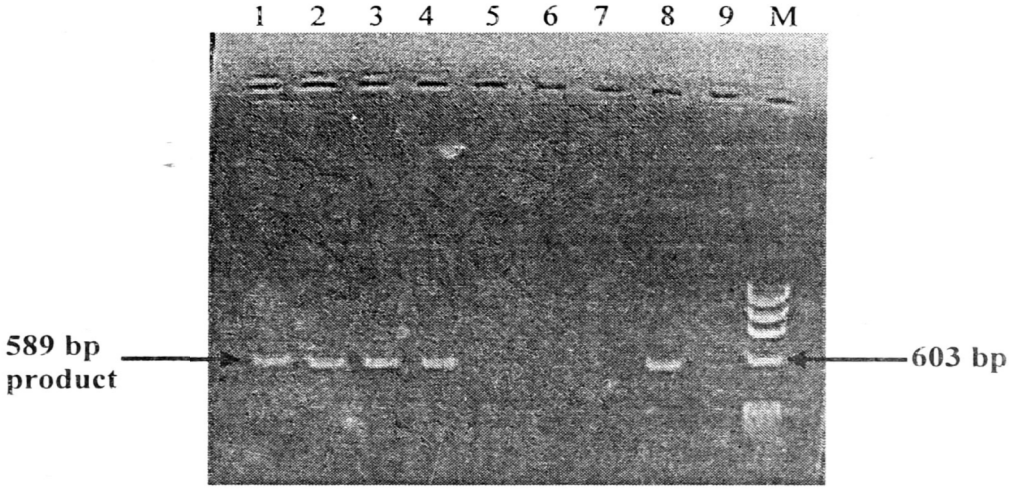

Figure 2. Amplified product of PCR using primer pair Mys F_1 and Mys R1 of different banana cultivars *in vitro* stage.
Lane 1-Ambul, 2-Kolikuttu 3-Seeni Kehel, 4-Pulathisi, 5-Dwarf Cavendish, 6-Anamalu, 7-Amban 8-Positive Control, 9-Negative control M-Molecular size Marker (Phi x 174. DNA/Hae III Helena Bio Sciences).

Table 1 summarizes the results obtained for the 12 different tissue cultured banana cultivars with different genomic configurations at three different growth stages, which were *in vitro* stage, hardening stage and in the field. Three different virus identification methods were employed as PCR, IC-PCR and visual observations of characteristic symptoms.

The target sequence of two primers were found with the PCR assay and the desired products were obtained in all the cultivars having genomic configuration B. In contrast to that cultivars with genome having only A were not amplified revealing the absence of the target sequence. In IC-PCR assay, no amplification was resulted in any of the cultivars tested and amplicons were found only in samples used as positive controls (Fig. 3). This result reveals the integration of the BSV genome in banana B genome. Similar findings were reported by Geering *et al.* (2005) for banana with A and B

genomes. Also the field established *in vitro* cultured plants did not express symptoms suggesting the absence of episomal infection.

Table 1. PCR and IC-PCR results of tissue cultured banana at PVIC, indexed for banana streak virus.

Cultivar	*Genomic configuration*	*In vitro stage*		*Hardening stage*		*Field*		*BSV symptoms expression*
		PCR	*IC-PCR*	*PCR*	*IC-PCR*	*PCR*	*IC-PCR*	
Dwarf Cavendish	AAA	(-)	(-)	(-)	(-)	(-)	(-)	N
Anamalu	AAA	(-)	(-)	(-)	(-)	(-)	(-)	N
Emban	AAA	(-)	(-)	(-)	(-)	(-)	(-)	N
Bin Kehel	AAA	(-)	(-)	(-)	(-)	(-)	(-)	N
Kandula	AAAB	(+)	(-)	(+)	(-)	(+)	(-)	N
Pulathisi	AABB	(+)	(-)	(+)	(-)	(+)	(-)	N
Nethrampalam	AAB	(+)	(-)	(+)	(-)	(+)	(-)	N
Ambul	AAB	(+)	(-)	(+)	(-)	(+)	(-)	N
Kolikuttu	AAB	(+)	(-)	(+)	(-)	(+)	(-)	N
Seeni Kehel	ABB	(+)	(-)	(+)	(-)	(+)	(-)	N
Ranel	ABB	(+)	(-)	(+)	(-)	(+)	(-)	N
Prasad	ABB	(+)	(-)	(+)	(-)	(+)	(-)	N

(-) Negative (+) Positive N- No BSV symptoms

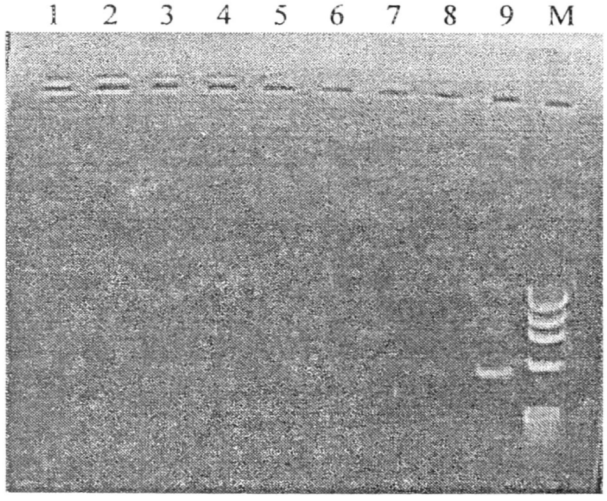

Figure 3. Amplified product of IC-PCR using primer pair Mys F1 and Mys R1 of different banana cultivars *in vitro* stage.
Lane 1-Ambul, 2-Kolikuttu, 3-Seeni Kehel, 4-Pulathisi, 5-Dwarf Cavendish, 6-Anamalu, 7-Amban, 8-Negative control, 9 Positive Control, M-Molecular size Marker (Phi x 174. DNA/Hae III Helena Bio Sciences).

For further confirmation of IC-PCR in detecting episomal BSV, samples with typical BSV symptoms were checked with both PCR and IC-PCR. In this case, cultivars with the B genome were obtained for screening

and all those samples were positive with PCR. More than 94% of the samples were positive with IC-PCR. This result suggests the suitability of IC-PCR for the detection of episomal BSV.

Further studies will be needed to investigate the integration and the potential activation of the BSV in banana genome.

Table 2. PCR and IC-PCR results of samples with typical BSV symptoms.

Cultivar	*No. of samples*	*PCR positive samples*	*% of PCR positives*	*IC-PCR positive samples*	*% of IC-PCR positives*
Ambul	36	36	100%	34	94.4%
Senni Kesel	21	21	100%	20	95.2%
Kolikuttu	12	12	100%	12	100%

CONCLUSIONS

In summary, the absence of episomal BSV in all the varieties tested at PVIC, displayed that it is not affected by tissue culture propagation of plant material which is done under optimum culture conditions using the following culture combination: culture media MS + BAP 5 mg/l for shoot multiplication, MS + 0.1 mg IAA for root growth and optimum sub culturing stage at 6^{th} sub culture.

It was found that at the molecular level, IC-PCR is the most appropriate methodology to detect episomal BSV in tissue cultured banana clones. Therefore, it can be successfully applied for the detection of induced form of BSV in tissue cultured banana clones. This information is extremely useful for the large scale screening of tissue cultured banana for episomal BSV in order to produce disease free planting material.

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