Identification of Strains of Banana Bunchy Top Virus Using Polymerase Chain Reaction (PCR)

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ABSTRACT

Banana Bunchy Top Virus (BBTV) is the most common and destructive disease of banana in Sri Lanka. This study was conducted with a view to identify different strains of Banana Bunchy Top Virus in the wet zone of Sri Lanka and study the relationship between symptom expression and different strains of BBTV. BBTV infected banana samples showing different levels of symptoms were collected from home gardens, small scale and large scale plantations in wet zone. The extracted nucleic acid of the virus was amplified by Polymerase Chain Reaction using four primer pairs. The presence of the virus was confirmed by using the BBTV genomic DNA specific primer pair, BBT1 and BBT2. Strains were identified following Su *et al.*, (1998) using the PCR banding patterns derived from three primer pairs C1-CR, S-CR and SR-CR which designated six PCR genotypes. Chi square analysis was used to observe the relationship between symptom expression and presence of BBTV and also the relationship between symptom expression and different BBTV PCR genotypes.

Chi square analysis showed a relationship between the presence of BBTV and severity levels of symptoms produced for dwarfing, short petioles and narrow leaves. There was no any relationship between symptom expression and the PCR genotypes of BBTV. Out of the six PCR genotypes characterized, only two PCR genotypes S-3 and My-S3 were detected in the wet zone of Sri Lanka. Fifty six percent of BBTV positive samples did not produce PCR amplification for any of the three primer pairs indicating the presence of unidentified strains.

KEYWORDS: Banana Bunchy Top Virus, BBTV Strains, PCR Genotypes, Wet Zone of Sri Lanka.

INTRODUCTION

The banana (*Musa spp.*) is an important fruit crop of the tropics. This crop is grown both as a staple fruit as well as a cash crop mainly for the local market. In 2003, the extent and production of banana in Sri Lanka were 49,255 ha and 393,384 Mt, respectively (Anon, 2004).

Banana plants are prone to number of virus diseases including Banana Bunchy Top Virus (BBTV), Cucumber Mosaic Virus (CMV), Banana Streak Virus (BSV) and Banana Bract Mosaic Virus (BBrMV). Among them BBTV has been the most common and destructive viral disease which hampered banana production in some Asian, Pacific regions. Outbreak of BBTV on several occasions during this century has become the limiting factor for the banana industry in Taiwan during 1900's, 1960's and 1980's (Su *et al.*, 1998). BBTV was first recorded in Sri Lanka in 1913 (Gadd, 1926).BBTV infection has been reported all around in Sri Lanka.

BBTV is a Nanovirus containing multiple, circular, ssDNA components (Harding *et al.*, 1991) which are associated with 18-20 nm virions (Dietzgen and Thomas, 1991). Burns *et al.* (1995) has revealed that BBTV has at least six components of ssDNA ranging sizes from 1018 to 1111 nucleotides in each. BBTV is transmitted persistently by the aphid *Pentalonia nigronervasa* but does not appear to replicate in its vector (Hafner *et al.*,1995). This virus is not mechanically transmissible and is apparently confined in phloem region.

Disease symptoms usually appear about a month after the infection. The initial symptoms of BBTV show dark green streak in the veins of lower portions of the leaf midrib and the petiole. They also show chlorosis, vein clearing, marginal necrosis and narrow leaves. The petioles are incompletely elongated. Shortening of internodes form a 'rosette' or 'bunchy top' appearance. Severely infected banana plants usually will not bear fruits, and if produced, the opening bunches are restricted or 'choked' by the pseudo stem. Studies on banana viruses revealed that BBTV infection at early stage causes 100% yield reduction.

Objective of the present study was to identify the different strains of BBTV in wet zone and study the relationship between symptom expression and different strains of BBTV. BBTV specific primer pair, BBT_1 and BBT_2 detects a specific complementary sequence of 350 bp of the BBTV genome. DNA components of the various BBTV strains have produced different PCR amplification patterns with the three primer pairs C1-CR, S-CR, and SR-CR used by Su *et al.*, (1998). All the three primer pairs produced a fragment of similar length of about 1.1 kb owing to the circular strands of virus genomic DNAs (1.1 kbs). The different PCR patterns were designated six PCR genotypes of BBTV S-2, S-3, MY-S3, I-1, M-1, L (Su *et al.*, 1998).

MATERIALS AND METHODS

This study was carried out at the Plant Virus Indexing Centre, Department of Agriculture, Gabadawatta, Homagama, from November 2004 to May 2005.

Sample Collection

BBTV infected leaf samples showing different levels of symptoms were collected from home gardens, small scale and large-scale plantations in wet zone.

The severity level of the symptoms, expressed as dwarfing, short petioles, chlorosis, vein clearing, narrow leaves, marginal necrosis and chocking was scored on a 0 - 4 scale for each symptom (Table 1). Considerable amounts of leaf pieces from the tip portion of newly emerged leaves, containing the mid rib were immediately used or preserved under -20 ^{0}C for later use.

Nucleic Acid Extraction

A protocol provided by Su, (1999) was followed for the extraction of nucleic acid. A leaf sample of 0.25 g was extracted with 1500 µl of DNA extraction buffer (100 mM Tris HCl, 100 mM EDTA, 250 mM NaCl pH 8.00) and 1% sarkosyl (N-Lauroyl sarcosine) (1350 µl of extraction buffer and 150 µl of 10% Sarkosyl). Tissue suspension was then stirred and transferred to a 1.5 µl eppendorf tube and incubated at 55 ^{0}C for 1 hour in a water bath. Tubes were then centrifuged at 6000 rpm for 5 minutes. After centrifugation, 400 µl of supernatant was collected. This was then incubated for 10 minutes at 65 ^{0}C after addition of 50 µl of 5M NaCl and 50 µl of CTAB (Hexadecryltrimethylammonium bromide)/NaCl (10% CTAB in 0.5 M NaCl). Two hundred and fifty µl of chloroform: isoamyl alcohol (24:1) was then added and mixed thoroughly and spun at 6000 rpm for 5 minutes.

Thereafter, 400 µl of supernatant was saved and 300 µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed thoroughly. Tubes were spun at 6000 rpm for 5 minutes. Three hundred µl aqueous suspension was saved and 0.6 volume of isopropanol was added to precipitate the Nucleic acid. Then the tubes were incubated at -20 ^{0}C overnight.

Tubes were then spun at 12,000 rpm for 15 minutes and the pellets were washed with 25 µl of 70% ethanol to remove CTAB residues. They were briefly dried and resuspended in 50 µl of TE buffer. The extract was then stored at -20 ^{0}C.

Polymerase Chain Reaction (PCR)

Primers;

The BBTV specific Primer Pair, BBT_1, 5' CTC GTC ATG TGC AAG GTT ATT GTC G 3' and BBT_2, 5' GAA GTT CTC CAG CTA TTC ATC GCC was used to amplify 350bp fragment (Harding *et al.*, 1993) from the virus genome.

Three primer pairs each producing a 1.1kb fragment (C1-CR, S-CR, and SR-CR), derived from common regions from sequences of DNA components of Taiwan severe strain such as stem loop (S-CR), right side of stem loop (SR-CR), left side of stem loop (C1-CR) (Su *et al.*, 1998). were also used. The sequences of three primer pairs are given below.

C1-CR:

F-5' GGA AGA AGC CTC TCA TCT GCT TCA GAG AGC 3'
R-5' CAG GCG CAC ACC TTG AGA AAC GAA AGG GAA 3'

S-CR:

F- 5' GGG GCT TAT TAT TAC CCC CAG C3'
R-5'AGC GCT TAC GTG GCG CAG CAC TAA CT3'

SR-CR:

F- 5' TGT CGT CGG CGA CGA AGT CG 3'
R-5' GGA CAT CCT CCT TCA GAA GAG AGA 3'

The PCR reaction mixture (25 µl) contained 13.0 µl of deionized water, 3.0 µl of 10xPCR buffer, 2.0 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs, 3.0 µl of primer C1-CR or S-CR or SR-CR (10 ng / µl) or 1.5 µl of (10 mM) BBT_1 and 1.5 µl of (10 mM) BBT_2 primer, 0.5 µl of (5 u / µl) Taq polymerase, 3.0 µl of DNA template (1;30 dilution of the extract).

The PCR amplification using BBT Primer pair was performed at 94 ^{0}C, 1 minute for 1 cycle; followed by 94 ^{0}C, 20 seconds; 60 ^{0}C, 1 minute; 72 ^{0}C, 2 minutes; for 35 cycles and final extension of 72 ^{0}C, 3 minutes

The PCR amplification using C1-CR primer was performed at 94 ^{0}C, 4 minutes; 50 ^{0}C, 1 minute; 72 ^{0}C, 2 minutes for 1 cycle; followed by 94 ^{0}C, 1 minute; 50 ^{0}C, 1 minute; 72 ^{0}C, 2 minutes ; for 30 cycles and final extension of 72 ^{0}C, 10 minutes.

The PCR cycles for S-CR and SR-CR Primer pairs were similar to the cycles for C1-CR primer pair except the annealing temperature which was at 60 ^{0}C. At the end of PCR amplification, samples were soaked at 4 ^{0}C for 3 hours.

Analysis of PCR Products

Amplified PCR products were analyzed by electrophoresis at 80V for 45 minutes in 1% agarose gel in TAE buffer (40 mM Tris HCl, 5 mM sodium acetate, and 1 mM EDTA pH 7.7 per litre) in the presence of 0.05 µg/ml ethidium bromide and photographed under ultra violet light (302 nm). ΦX 174 RF DNA / Hae 111 marker was used as the DNA molecular marker.

Statistical Analysis

The relationship between symptom expression and different strains of BBTV and the relationship between severity levels of each symptom and presence of BBTV were analyzed using chi-square analysis (Kothari, 1985).

RESULTS AND DISCUSSION

In majority of the samples, it was observed that BBTV affected plants have shown complex of symptoms. Dwarfing, short petioles, chlorosis, vein clearing, narrow leaves, marginal necrosis, chocking were characterized in the samples collected, as shown in the table 1. Each symptom was scored 0 – 4 severity scale as absence of symptom-0, mild symptom-1, moderate symptom -2, severe symptom-3 and very severe symptom-4. The overall disease symptom expression of collected samples, showed disease index values, ranging from 1 – 7 which indicate the number of symptoms present in each sample.

Short petioles, chlorosis and vein clearing were observed as the most common symptoms in the infected plants having total score of 46, 42 and 53 respectively (Table 1).

Table 1. Symptom expression of collected samples.

Sample Number	Dwarf-ness	Short Petioles	Vein Chloro-sis	Vein Clearing	Narrow Leaves	Marginal Necrosis	Chock-ing	Total Score	Disease Index	Presence of Virus	Strain
1	0	0	1	1	0	0	1	3	3	+	S-3
2	0	1	1	1	1	1	1	6	6	+	My-S3
3	3	2	3	1	3	3	0	15	6	+	S-3
4	2	3	2	2	2	0	0	11	5	+	S-3
5	0	0	1	0	0	1	0	2	2	-	_
6	3	3	1	3	2	0	1	13	6	+	My-S3
7	0	1	0	1	0	0	0	2	2	+	My-S3
8	1	2	1	2	0	1	0	7	5	+	My-S3
9	1	1	1	1	1	1	0	6	6	+	S-3
10	0	2	1	3	0	0	1	7	4	+	My-S3
11	1	2	1	3	2	2	0	11	6	+	S-3
12	0	0	1	1	0	0	0	2	2	-	_
13	0	1	3	3	2	3	1	13	5	+	My-S3
14	2	3	3	2	2	2	3	17	7	+	Other
15	0	1	3	2	0	1	1	8	5	-	_
16	0	1	2	1	1	0	0	5	4	-	_
17	0	1	0	3	0	0	1	5	3	-	_
18	4	4	3	2	3	1	4	21	7	+	Other
19	0	0	1	3	0	0	0	4	2	-	_
20	2	3	3	3	2	2	1	16	7	+	Other
21	1	2	2	2	3	3	1	14	7	+	Other
22	0	0	0	0	0	0	1	1	1	-	_
23	3	2	1	2	0	2	1	11	6	+	Other
24	0	1	0	1	0	0	1	3	3	+	Other
25	0	1	0	1	1	1	0	4	4	+	Other
26	0	0	2	1	1	1	0	5	4	+	Other
27	0	0	0	1	0	0	0	1	1	-	_
28	2	2	2	3	2	3	1	15	7	+	Other
29	2	2	1	1	3	0	3	12	6	+	Other
30	1	0	1	0	3	0	0	5	3	+	Other
31	1	1	0	0	1	0	1	4	4	+	Other
32	0	1	0	1	2	0	2	6	4	+	Other
33	2	3	1	2	2	0	3	13	6	+	Other
Total score	31	46	42	53	39	28	29				

0_ absence of symptom 1_ Mild symptom expression 2_ Moderate symptom expression 3_ Severe symptom expression 4_ Very severe symptom expression

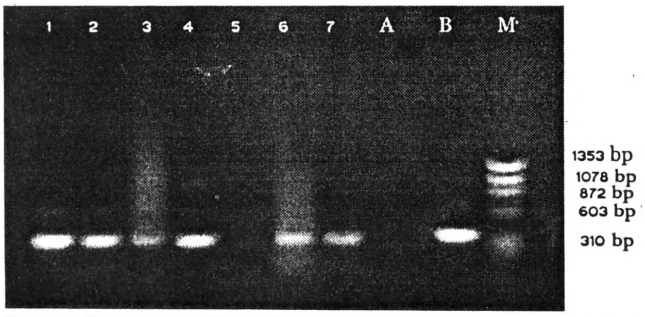

Plate 1. Detection of BBTV by PCR with respect to BBT_1 and BBT_2 primer pair followed by electrophoresis analysis resulting a 350bp fragment. M- ΦX 174 DNA / Hae 111 marker, A-Negative control, B- Positive control, 1, 2, 3, 4, 6 and 7- BBTV detected samples, 5- BBTV undetected sample.

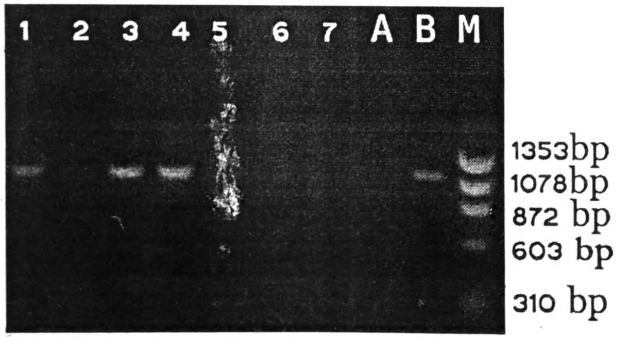

Plate 2. Detection of C1-CR primer sequence in BBTV DNA by PCR with respect to C1-CR primer pair followed by electrophoresis analysis resulting a 1.1bp fragment. M- ΦX 174 DNA / Hae 111 marker, A- Negative control, B- Positive control, 1, 3, and 4- Positive samples for C1-CR primer pair, 2, 5, 6 and 7- Negative samples for C1-CR primer pair.

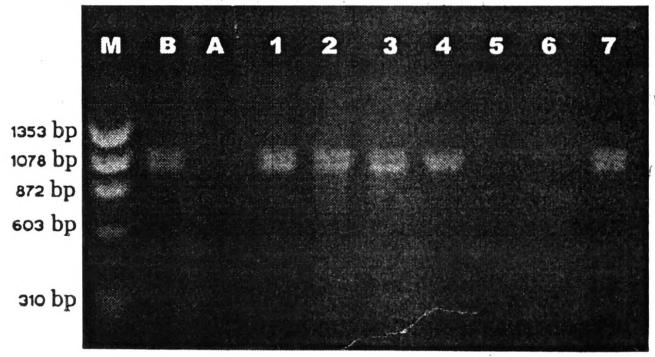

Plate 3. Detection of S-CR primer sequence in BBTV DNA by PCR with respect to S-CR primer pair followed by electrophoresis analysis resulting a 1.1bp fragment. M- ΦX 174 DNA / Hae 111 marker, A-Negative control, B-Positive control, 1, 2, 3, 4 and 7- Positive samples for S-CR primer pair, 5 and 6 - Negative samples for S-CR primer pair.

Table 2. Detection of BBTV genotypes by PCR using different primers.

Primer Pairs				Number of samples	Strain Isolate of BBTV
BBT1/ BBT2	C1-CR	S-CR	SR-CR		
+	+	+	-	5	S-3
+	-	+	-	6	MyS3
+	-	-	-	14	Other
-	-	-	-	8	-
25	5	11	0	33	

+ Amplified product present
- Amplified product absent

C1-CR, S-CR, and SR-CR primer pairs were derived from common regions among sequences of DNA components of Taiwan severe strain (Su *et al.*, 1998). The presence of 1.1 kb band for any of above primers, confirms the presence of a particular complementary sequence of template DNA strand in each specific strain. Hence, DNA of various BBTV strains generates different amplification patterns with respect to above three primers reflecting the sequence variation of each strain genome. Su *et al.*, (1998) have identified six PCR genotypes (Type I to VI) based on different PCR patterns obtained from BBTV strains (Table 3). Three representative isolates of severe strain, S-2, S-3 and My-S3 were differentiated into three different PCR genotypes. Isolate S-2 being amplified with the three primer pairs was designated to type 1; S-3 reacting with C1-CR and S-CR primers belonged to type II; and My- S3 reacting with S-CR primer only, designated to type III (Su *et al.*, 1998).

Table 3. Differentiation of BBTV strains by PCR with different primer pairs, showing six genotypes of virus strains in amplification patterns (*Su et al.*, 1998).

Primer Pairs	Strain Isolate of BBTV [a]					
	S-2	S-3	My-S3	I-1	M-1	L
	PCR genotype					
	I	II	III	IV	V	VI
C1-CR	+++	+++	_	+++	+++	+[c]
S-CR	+++	+++	+++	-	++/+[b]	-
SR-CR	++	-	-	++	++	-

(a) S: Severe, I: Intermediate, M: Mild, L: Latant
(b) 1kb/ 0.5kb
(c) The different PCR amplification patterns shown by electrophoresis analysis of PCR products in agarosegel:-, no amplification; relative amount of positive amplification shown by intensity of electrophoretic bands of DNA products + low, ++ moderate, +++ high products.

The PCR amplification patterns, generated by the BBTV DNA collected from the locally grown banana using Su's primer pairs are given in the table 2. Following the PCR banding pattern described by Su *et al.*, (1998) (Table 3), it was observed the presence of type II and type III PCR genotypes from the locally collected samples. These two genotypes (type II and type III) represent the two severe strain isolates of BBTV, *i.e.* S-3 and My-S3, respectively.

Fourteen BBTV infected samples, though were confirmed for the presence of virus by BBT_1, BBT_2 primer pair, have not resulted any amplified product by the three Su's primer pairs. This indicates the absence of complementary sequences in the BBTV DNA of these samples for the Su's primer sequences. It is therefore, possible for the existence of different strains of BBTV in the locally cultivated banana from the six PCR genotypes identified in Su *et al.*, (1998). Further studies are necessary to recognize these unidentified strains.

Relationship between symptom expression and the presence of BBTV.

There was no relationship between symptom expression and different strains of BBTV. The seven symptoms associated with the BBTV infection were observed for the symptom expression levels and the presence of BBTV using chi square analysis. The probability value of chi square indicated that there was a significant relationship between the presence of BBTV and severity levels of dwarfing, short petioles and narrow leaves (Table 4).

Chi square analysis showed that all the samples having 1, 2, 3 and 4 severity levels of dwarfing were infected by the virus (Table 5).

Table 5. Relationship between severity of dwarfing and presence of BBTV.

Severity level	Percentages	
	Positive for BBTV	Negative for BBTV
0	52.94	47.06
1	100.00	0.00
2	100.00	0.00
3	100.00	0.00
4	100.00	0.00

N = 33 samples, prob = 0.0415 (< 0.05), chi square value = 9.9388

All the plants showing the expression of short petioles at the severity levels 2, 3 and 4 were infected by the virus (Table 6). Only 72% of the plants expressing at severity level 1 were shown to be infected by the virus.

Chi square test indicates that all the plants giving narrow leaves symptom at the severity level 2 and 3 were shown to be infected by the virus. Among the level 1 severity group, 16.67% plants were shown to be virus free.

Table 6. Relationship between severity of short petioles and presence of BBTV.

Severity level	Percentages	
	Positive for BBTV	Negative for BBTV
0	37.50	62.50
1	72.73	27.27
2	100.00	0.00
3	100.00	0.00
4	100.00	0.00

N = 33 samples, prob = 0.0276 (< 0.05), chi square value = 10.9106

Table 4. Results of the chi square test for the relationship between symptom expression and the presence of BBTV.

Symptoms	Dwarfing	Short petiole	Chlorosis	Vein Clearing	Narrow leaves	Marginal necrosis	Chocking
Chi square value	9.9388 *	9.9388 *	1.0625^{NS}	2.0577^{NS}	10.8710 *	3.6931^{NS}	2.6636^{NS}

* - *Significant ($p < 0.05$)* NS - *Not Significant*

Table 7. Relationship between severity of narrow leaves and presence of BBTV.

Severity level	Percentage Positive for BBTV	Negative for BBTV
0	46.15	53.85
1	83.33	16.67
2	100.00	0.00
3	100.00	0.00
4	0.00	0.00

N = 33 samples, prob = 0.0124 (< 0.05), chi square value = 10.8710

CONCLUSIONS

The observations made in this study have proved that S-3 and My-S3 severe strains of BBTV were present in some of the samples in the wet zone of Sri Lanka. The other BBTV positive strains, which were not identified, by Su's primers, have to be characterized in future experiments.

The results showed that short petioles, chlorosis and vein clearing are the most common symptoms produced by the BBTV. Expression of the symptoms related to dwarfing, short petioles and narrow leaves at 2, 3 and 4 severity levels confirms the presence of BBTV.

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