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Detection of banana bunchy top virus isolates from Malaysia using the polymerase chain reaction

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Abstract

The presence of banana bunchy top virus (BBTV) which causes severe economic losses has been tested in samples collected from Malaysia using polymerase chain reaction method. Leaf and mid rib tissues collected from research stations and farmers' fields in Malaysia were received at Queensland University of Technology in March 1996 and stored at -20^0 C and tested in October 1996. The protocol and the methodologies are described. The results indicate that 18 out of the 52 tested samples were positive for BBTV. The results varied from strong, moderate to mild reactions. Both apparently diseased as well as apparently healthy field samples gave certain percentages of positive reaction. These tests confirmed the presence of BBTV in certain parts of Malaysia.

Key words: BBTV PCR

1. Introduction

Banana bunchy top virus (BBTV) causes an important disease in bananas which hamper banana production in many banana growing countries. The etiology of this disorder is unclear. Earlier thinking was that BBTV was caused by a Single Stranded RNA virus (Dale *et. al*, 1986, Iskra *et al* 1989, Su & Wu, 1989). More recently ssDNA has been isolated from virions purified from diseased banana plants in Australia, Hawaii, Indonesia, Tonga, Pakistan, Brudi, Egypt, India, Western Samoa, Philippines, Taiwan and Vietnam. (Harding *et al,* 1991, Dietzgen and Thomas 1991, Karan *et al,* 1994, Khalid & Soomro, 1993). Further cloning and sequencing by Burns (1995), revealed that BBTV has at least Six components of ss DNA all of which were identified in every BBTV isolate.

Polymerase chain reaction (PCR) which involves the enzymatic amplification of a DNA fragment defined by two oligonucleotide primers have been used to diagnose a number of plant viruses (Navot *et al* 1992, Robertson *et al* 1991, Rybicki and Hughes 1990).

The work reported in this paper is the outcome of a research project initiated with the objective of detecting BBTV in banana samples received from Malaysia.

2. Materials and methods

Banana samples consisting of leaf and mid-rib tissues were received from Pusat Penyelidkan Pertanian Agricultural Research Centere, Rembus, Tarat and from farmers' fields in Asojaya/Sadang Jaya in Malaysia. These were received in March 1996 at Queensland University of Technology in Australia and were stored at-20 °C in the Centre for Molecular Biotechnology laboratories. Analysis were done in October 1996 and the details of the samples assayed are presented in Table I.

Thomas, M. Burns, Robert M. Harding and James L. Dale (1995). "The genome organization of banana bunchy top virus. Analysis of six ssDNA components. J. of Gen. Virology 76: 1471-1482 pp.

Table 1: Banana samples from Malaysia Included in Polymerase chain reaction tests.

Sample numbers								Description
S:	17,	18,	19,	20,	21,	22,	23,	Samples collected from infected
	24A,	24B,	25,	26,	27,	28,		cultivations Symptoms ranged from:
	29A,	29B,	30A	,30B,	31,	32,		slight yellowing, interveinal
	33,	34,	35,	36,	37,	38,	39	chlorosis, bunch narrow leaves.
	40,	41,	42,	43,	44,	45,	46,	
	47,	48.						
S:	49,	52,	54,	55,	56			Samples from apparently healthy looking plants from Asajaya.
S:	64,	69,	71,	74,	86,	98.		From Tarat
S:	105,	110,	118,	122,	125,	128.		From Rembus

Nucleic acid preparation

Nucleic acid was extracted from infected and apparently healthy banana tissue using Su' s protocol (Personal communication) About 0.1-0.2 g of tissue were ground into a fine powder by using liquid nitrogen in a mortar with pestle. The frozen powder was then transferred to a beaker and 2 ml of BBTV extraction buffer (consisting of Tris base 1. 18g, Tris HCI 6.35g, Sodium N,N Diethiocarbamate tri-hydrate 0.1%, sucrose 5% 0.5% skimmed milk pH 7.4, per litre) was immediately added and centrifuged at 10,000 rpm for 10 minutes.

After centrifugation the aqueous phase was transferred to a micro-centrifuge tube and an equal volume of DNA extraction buffer consisting of 0.02M Tris pH 9, 1 mM EDTA,4% SDS, was added and thoroughly mixed. Thereafter 175 µl of 5M Na Cl per 1ml of supernatant and 140 µl of 1.5% Catyl trimethyl ammonium bromide/ 1.5 M Na Cl per 1 ml of supernatant were added, vortexed and spun at 10,000 rpm for 10 minutes. The supernatant was removed after centrifugation and an equal volume of chloroform/ isoamyl alcohol 24:1 (v/v) was added to give a 1:1 v/v ratio, vortexed and centrifuged at 10,000 rpm for 10 minutes. Isopropanol (0.6 volume) was then added to the supernatant which was then kept at -20° C for nuclelic acid precipitation. Precipitated nucleic acid was collected by centrifugation at 10, 000 rpm for 10 minutes. The pellet was washed with 70% ethanol to remove any residual CTAB, driefly dried and 100 µl of double distilled water was added. These were then stored at- 20° C till further use.

Primers

Two oligonucleotide primers

FPCR4-5' TTCCCAGGCGCACACCTTGAGAAACGAAAG 3'
and

30 mer-5' GGAAGAAGCCTCTCATCTGCTTCAGAGAGC 3'

were used for the amplification of component 1 of BBTV Which yielded 1 Kb product (Thomas & Dietzygen,1991).

PCR

For PCR each sample contained 5 µl of PCR buffer (10 x PCR buffer 11, Perkin Elmer), 3µl of $MgCl_2$ (25 mM, 1.5ml), 1 µl of 10 mM dNTPS (Pharmacia Biotech), 1.5 µl each of primers (20 pmol/µl,) 0.2 µl of Taq polymerase (5µ/ml, Perkin Elmer) and 1 µl of DNA (from 1:10 dilutions) from banana. De- ionized water was added to give a final volume of 50 µl. The mixtures were incubated in a DNA Thermal cycler (Hobbes) at 94C° for 4 min followed by 45 cycles of 1 min at 94°C 1 min at 35°C and 2 min at 72°Cand final extenslon of 72°C for 10 miu.

Five micro litres of amplified PCR products were separated on a 1% agarose gel (AGP Technologies, Hi- Pure low EEC Agarose) in TAE (40 mM Tris H C L, 5 mM sodium acetate, 1mM EDTA pH 7.7) buffer with 200 µg/ml ethidium bromide. Gels were electrophoreses at 80 V for 45 mins and

stained with ethidium bromide (200μg/ml). DNA molecular weight marker IX was used as size marker and PCR products were visualized on a UV transillumintor and photographed using polaroid film.

3. Results

BBTV detection by PCR

Banana Bunchy top Virus was detected in 18 samples out of 52 samples assayed (Table 2, Figure 1.). Strong reactions were obtained from the samples of S 21, S 24A, S 24B, S 25B, S 31, and S 48. which were collected from a severely infected field. Weak products were also obtained from the same field in samples S 34, S 35, S 52, and S 54. Interestingly very strong PCR reactions were obtained from S 74 and S.86 collected from apparently healthy looking plants in Tarat Station. Moderate to mild products were given by S 64, S 98 S 110, S 118, S 122 and S 125.

Table 2: Detection of banana bunchy top virus Malaysian isolates in banana by Polymerase Chain Reaction

Sample number	Intensity of PCR reaction**
S 21	+ + +
S 24A	+ + +
S 24B	+ + +
S 25B	+ + +
S 31	+ + +
S 34	+ +
S 35	+
S 48	+ + +
S 52	+
S 54	+ +
S 64	+ +
S 74	+ + +
S 86	+ + +
S 98	+ +
S 110	+
S 118	+ +
S 122	+ +
S 125	+ +

**Key : +++ = intense reaction
++ = moderate reaction
+ = mild reaction

Samles without reaction were: S17, 18, 19, 20, 22, 23, 26, 27, 28, 29A, 29B, 30A, 30B, 32, 33, 36, 37,38, 39, 40, 41, 42, 43, 44, 45, 46, 45, 46, 47, 49, 52, 55, 69.

Figure 1: Results of Polymerase chain reaction tests of BBTV (Malaysia) taken from leaf material using Primers (FPRC 4 and 30 Mer F3). PCR products were run of 1‰ agarose gel and stained with bromide.

The latter 4 samples were collected from another research stationnamed Rembus. A not eworthy feature was that S 98 sample was collected. from an apparently healthy looking wild banana plant.

Other samples gave negative results despite several tests.

4. Discussion

Banana bunchy top virus DNA sequence can be reliably amplified by PCR from infected bananas eventhough the samples were stored at-20^0 C for over 7 months. The primer pair gave the best results in reaction with the temperature of the annealing phase at 35^0 C. It was possible only to detect 25% diseased samples out of 35 samples collected from a diseaded field by doing PCR. Nevertheless, 53% of diseased samples were detected out of 17 samples assayed from apparently healthy looking plants. The efficiency of virus detection by PCR could be influenced by several factors in which freshness of the tissues could be considered as one. Polymerase chain reaction confirmed the presence of BBTV in Malaysia and it is more useful for the detection of BBTV in any rapid multiplication programmes and in quarantine services.

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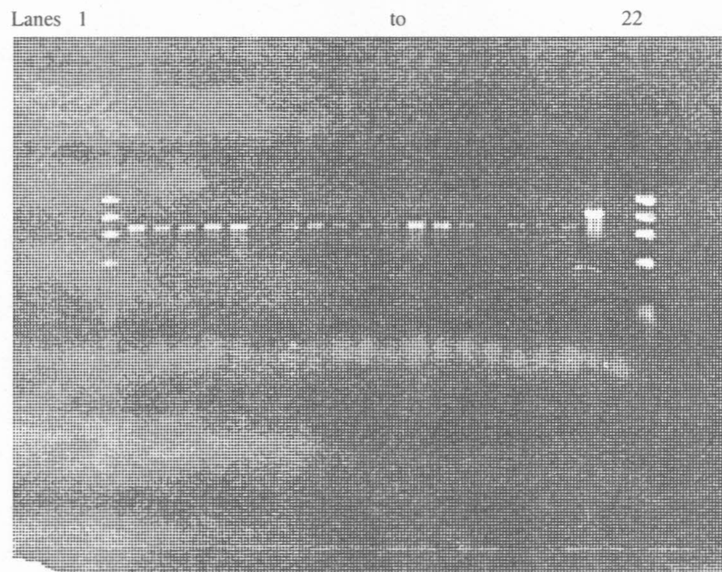
Figure 1: Results of Polymerase chain reaction tests of BBTV (Malaysia) taken from leaf material using Primers (FPRC 4 and 30 MER F3).PCR products were run on 1‰ agarose gel and stained with ethidium bromide.

Lanes 1 to 22



Lane	1	-	λ	DNA marker
Lane	2	-	S	21
Lane	3	-		24A
Lane	4	-		24B
Lane	5	-		25
Lane	6	-		31
Lane	7	-		34
Lane	8	-		35
Lane	9	-		48
Lane	10	-		52
Lane	11	-		54
Lane	12	-		54
Lane	13	-		64
Lane	14	-		74
Lane	15	-		86
Lane	16	-		98
Lane	17	-		110
Lane	18	-		122
Lane	19	-		125
Lane	20	-	Plasmid contorl	
Lane	21	-	negative control	
Lane	22	-	DNA Markel	