

RFLP-BASED RELATIONSHIP OF PAKISTANI ISOLATE OF BANANA BUNCHY TOP VIRUS WITH SOUTH PACIFIC VIRUS GROUP

**TAHIRA YASMIN, S.M. SAQLAIN NAQVI*, HUSSAIN SHAH
AND SAIF KHALID**

*Plant Virology Laboratory, Crop Diseases Research Program, National Agricultural
Research Centre, Islamabad, Pakistan and *Department of Biochemistry,
University of Arid Agriculture, Rawalpindi, Pakistan*

Abstract

Restriction Fragment Length Polymorphism (RFLP) technique was employed to investigate the relationship of Pakistani isolates of banana bunchy top virus (BBTV) with Asia and South Pacific strains. The PCR amplified product of BBTV DNA component I was digested with nine different restriction endonucleases (Bam HI, EcoRI, EcoRV, HaeIII, HincII, HindIII, PstI, RsaI and SmaI) and analyzed by agarose gel electrophoresis. HaeIII resulted in three DNA fragments of 505, 232 and 188 base pairs while RsaI gave four fragments of 310, 310, 250 and 200 base pairs. However, the DNA remained undigested with rest of the endonucleases. On the basis of electrophoretic patterns of restriction fragments, Pakistani BBTV isolate is closely related to South Pacific virus group.

Introduction

Banana Bunchy Top Virus Disease (BBTV) transmitted by an aphid (*Pentalonia nigronervosa*) threatens banana (*Musa* spp.) cultivation; throughout the world (Dale, 1987). The disease was first reported from Fiji in 1889 and later confirmed in the continents of Asia, Africa and Australia as well as in Pacific islands but not in America and Israel. BBTV is isometric and multicomponent particles 18-20 nm in diameter and multi-component genome is divided in nine cssDNA components of 1Kb each. A single coat protein weighs about M_r 20,500 Da. It belongs to a recently recognized "nanavirus" group. The disease is characterized by chlorosis, dark green streaks on leaf petiole, midrib and pseudostem and bunchy appearance (Fig. 1) as reported by Burns *et al.*, (1995), Smith *et al.*, (1998) and Yasmin (2001).

Banana an important tropical fruit crop of Pakistan is cultivated over an area of 31,200 ha with a production of 149,700 tonnes per years (Anon., 2002). Sindh province is the major banana-growing region with 87% of the total area under cultivation. The plantations are more concentrated in districts of Thatta, Badin, Hyderabad, Mirpurkhas, Sanghar, Nawabshah, Naushero-feroze and Khairpur. A disease of unknown etiology was observed in 1989 in the coastal regions of Sindh that has caused more than 50% yield losses within two years of disease epidemic during 1990-92. The disease was identified as BBTD in 1991 (Khalid *et al.*, 1993). Disease monitoring data shows that it is spreading slowly from southern districts towards northward and will possibly spread to the entire disease free areas (Fig. 2) within few years (Yasmin, 2001).

BBTV isolates occurring in different countries are known to be serologically identical. Moreover, on the basis of nucleotide sequence data of DNA component 1, two virus groups have been identified as South Pacific, prevalent in Australia, Hawaii and some Pacific Islands and Asian, occurring in China, Taiwan and Southeast Asia (Dale *et al.*, 1998; Beetham *et al.*, 1999). During the present study, restriction fragment length polymorphism (RFLP) technique was employed to identified the relation of Pakistani isolates of BBTV with internationally recognized BBTV groups.



Fig. 1. Symptoms of BBTD on banana plants.

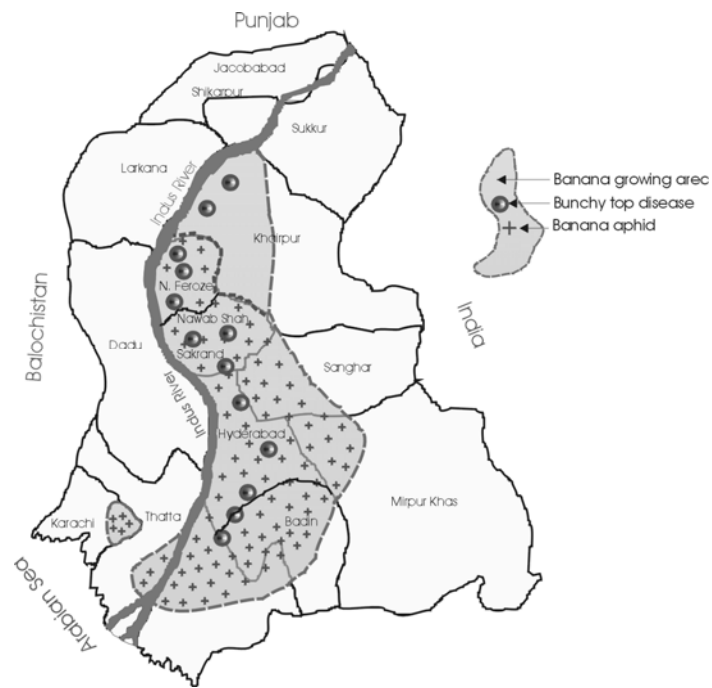


Fig. 2. Banana-growing, BBTV-infected areas and banana aphid distribution in Sindh, Pakistan

Materials and Methods

Source of plant material: Infected BBTV samples were collected from six major banana-growing areas of Sindh province viz., Karachi, Thatta, Badin Hyderabad, Nawabshah and Nausher-feroze) and were processed for polymorphism study.

DNA extraction: Total DNA was extracted from healthy and BBTV-infected leaves from each sample employing the following methods:

Method I: Genomic DNA was extracted following the method of Karan *et al.* (1994) using 1g each of healthy and diseased tissues. Extracted DNAs were subjected to enzyme digestion with DNase-free RNase (1 μ g/ μ l) and RNase-free DNase (0.2 μ g/ μ l) at 37°C for 1 hour and analyzed by agarose gel electrophoresis.

Method II: DNA was extracted following the method of Gawel & Jarret (1991) with some minor modifications. Banana tissues (healthy and diseased) were triturated in liquid nitrogen and 20 ml of extraction buffer (0.2% CTAB, 0.5 M EDTA, 1.4 M NaCl, 0.5M Tris and 0.1% 2-mercaptoethanol), pre-warmed at 65°C, was added. The mixture was mixed well, incubated at 65°C for 1 hour followed by addition of an equal volume of chloroform: isoamyl (24:1) and invert mixed for 15 minutes. The suspension was centrifuged at 7,000 rpm for 5 minutes at room temperature and the resulting aqueous phase was filtered through muslin cloth discarding the organic phase. This step was repeated once. An equal volume of isopropanol was added to the mixture until DNA precipitated and centrifuged at 10,000 rpm for 10 minutes. DNA pellet was washed in 70 % ethanol, blot dried, suspended in 50 μ l of TE buffer (10 mM Tris, 1 mM EDTA, pH=8.00) and stored at 4°C. Extracted DNAs were subjected to enzyme digestion with RNase and DNase and analyzed by agarose gel electrophoresis.

Method III: In this method DNA was extracted from healthy and BBTV-infected banana leaves using QIAprep Spin, Miniprep Kit of Germany.

Agarose gel electrophoresis: DNA preparations were analyzed on horizontal mini agarose gel electrophoresis apparatus. Agarose (1%) was dissolved in Tris-Borate-EDTA (TBE) buffer (0.5:0.045M Tris Borate, 0.001M EDTA) by heating for 1 minute, poured onto the gel template and solidified for 10-30 minutes at room temperature. After polymerization, comb was removed and tank buffer (0.5x TBE) was added to the gel tank. Samples (15 μ l each) along with the standard (1Kb DNA ladder) were mixed separately with 5 μ l of tracking dye i.e. bromophenol blue ((0.25% Bromophenol blue, 0.25% Xylene Cyanol FF, 30% Glycerol) and loaded in duplicate. The voltage was adjusted to 45 mA and 75 V for 1.5 hours. On completion of gel run, it was stained in 0.02% (w/v) Ethidium Bromide solution for 30 minutes and washed in distilled water 3-4 times. Gel was viewed over UV trans-illuminator at 300 nm and photographed.

DNA quantitation: The extracted DNAs were quantified in the following way:

DNA concentration: To determine concentration of extracted DNA, samples (extracted by Method I & II) were diluted 200 times in distilled water. Reference was set with distilled water and O.D was determined at a wavelength of 260 nm. From O.D, DNA concentration in μ g/ μ l of each sample was determined. DNA with high concentration was processed for further analysis.

Purity of DNA: To assess the purity of DNA samples (extracted by Method I & II), ratio of $A_{260\text{nm}}: A_{280\text{nm}}$ was calculated and purity of the extracted DNAs were determined. DNA with high purity was processed further.

Polymerase chain reaction (PCR): All DNA samples (healthy and diseased) were processed separately for amplification of BBTV DNA component 1.

PCR primer design: For BBTV DNA component 1, a pair of oligonucleotide primers was designed from known BBTV sequence reported by Xie & Hu (1995) having the following sequences;

Component 1 Forward	5'-GAATGGAATAATTCAAAGCGG-3'	(854-874)
Reverse	3'-ACAGTCTACATATATCCAATG-5'	(771-751)

Amplification and analysis: Each PCR reaction was carried out in 50 μl mixture of 10x PCR buffer (10mM Tris. HCl pH=9.00, 50 mM KCl, 1.5 mM MgCl_2 , 0.1% Triton X-100), 0.25 mM deoxyribonucleotide triphosphates (dNTPs), 2.5 units of Taq DNA Polymerase, 0.4 μM each of forward and reverse primer. One μl of template DNA was used per 50 μl PCR reaction. The following cycling conditions were tried for amplification of DNA component 1:

- (1) Denaturation at 94°C for 3 min. annealing at 50°C for 2 min., extension at 72°C for 3 min. followed by 40 cycles at 94°C for 1 min. 50°C for 1 min. 72°C for 2 min. and final extension at 72°C for 7 minutes,
- (2) Denaturation at 94°C for 4 min. annealing at 37°C for 2 min., extension at 72°C for 3 min. followed by 30 cycles at 94°C for 1 min. 37°C for 1 min. 72°C for 3 min. and final extension at 72°C for 10 minutes,
- (3) Denaturation at 94°C for 3 min. annealing at 40°C for 2 min., extension at 72°C for 3 min. followed by 40 cycles at 94°C for 1 min. 40°C for 1 min. 72°C for 3 min. and final extension at 72°C for 7 minutes.

Optimized conditions of PCR were repeated at least three times. The amplified products were analyzed by horizontal agarose gel electrophoresis as described above.

Restriction fragment length polymorphism (RFLP): PCR product of each sample was re-precipitated separately by adding 2.5 volume 100% ethanol and 0.1 volume 3M sodium acetate. Digestion of PCR product was made using nine different restriction endonucleases (Bam HI, EcoRI, EcoRV, HaeIII, HincII, HindIII, PstI, RsaI and SmaI). The reaction mixture of each single digest was prepared by adding 2 μl of amplified DNA component 1 (containing about 100ng of PCR product) to 0.5 μl of 10U/ μl of restriction enzyme and 2 μl of 10x enzyme buffer. The volume of reaction mixture was maintained to 20 μl with sterile distilled water and incubated at 37°C for 2 to 3 hours in circulating water bath. Digestion of each sample with the above mentioned restriction enzymes were repeated thrice. The restriction pattern of each sample was compared among themselves as well as with the two known BBTV groups.

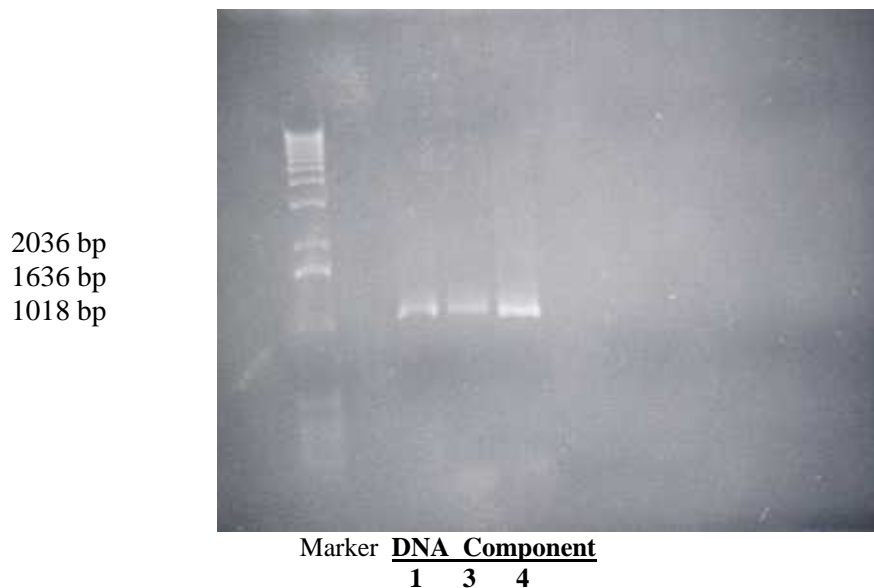


Fig. 3. Amplified DNA component 1, 3 and 4 (DNA extraction by CTAB method).

Analysis: The digested products were analyzed by agarose gel electrophoresis using 1% and 1.5% agarose gel as mentioned earlier and was repeated three times.

Results and Discussion

Nucleic acid analysis: CTAB and QIA miniprep Kit methods of DNA extraction gave DNA concentration of $0.89\mu\text{g}/\mu\text{l}$ and $0.95\mu\text{g}/\mu\text{l}$ with purity of 1.69 and 1.82 respectively. QIA method has yielded 0.13 better quality DNA than method II (CTAB). Thus extraction through kit was easy, quick and efficient as compared to CTAB method, which needs careful handling during separation of various phases. DNAs extracted through CTAB method was treated with RNase while that of kit method was mostly found free of cellular RNA as analyzed by agarose gel electrophoresis. DNase treatment removed DNA bands sometimes leaving a smear at the end of gel while RNase removed host RNA smears leaving unaffected viral DNA as obvious from Fig 3.

In the present findings, amplification of BBTV DNA component 1 was successful from total genomic DNA. These results are consistent with findings of Xie & Hu (1995) and Hu *et al.* (1996). The three DNA extraction methods employed gave varied quality of preparations. Gawel & Jarret (1991) extraction method produced comparatively better concentration ($0.89\mu\text{g}/\mu\text{l}$) and purity (1.69) while QIA method gave higher concentration ($0.95\mu\text{g}/\mu\text{l}$) and quality (1.82) of DNA.

PCR: Amplification of BBTV DNA was successful using the following cycling conditions i.e. denaturation at 94°C for 3 min. annealing at 50°C for 2 min. extension at 72°C for 3 min. followed by 40 cycles at 94°C for 1 min. 50°C for 1 min. 72°C for 2 min. and final extension at 72°C for 7 minutes, without changing the annealing temperature. The amplified product gave a single DNA fragment of about 1Kb (Fig. 3). No non-specific band was observed in any amplified DNA.

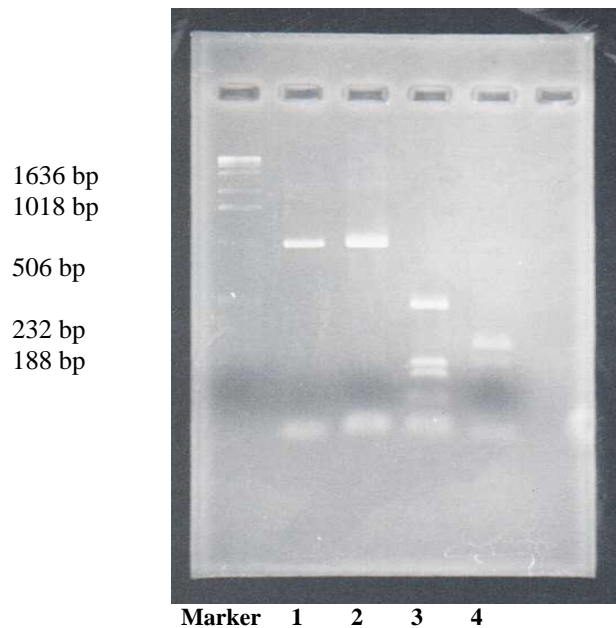


Fig. 4. RFLP-analyzed restriction fragments of BBTV DNA Component 1 digested with EcoRI (1), EcoRV (2), HaeIII (3) and RsaI (4).



Fig. 5. RFLP-analyzed restriction fragments of BBTV DNA Component 1 digested with BamHI (1), HincII (2), HindIII (3), HaeIII (4), PstI (5) and SmaI (6).

Standardization of PCR technique may help to detect and characterize BBTV at molecular level in turn helping to enhance the detection capabilities. Using this sensitive technique, few molecules of viral DNA can be easily detected not only in plant samples but also in individual viruliferous aphid and latent hosts of BBTV as reported by Hu *et al.* (1996).

Polymorphism in Pakistani BBTV isolates: Digestions of PCR products of DNA (from each DNA sample) with HaeIII resulted in three DNA fragments of 505, 232 and 188 base pairs with a total of 925 base pairs while four fragments of 310, 310, 250 and 200 base pairs were obtained with RsaI giving a total of 1070 base pairs. However, DNA remained undigested with BamHI, EcoRI, EcoRV, HincII, HindIII, PstI and SmaI endonucleases (Fig. 4 & 5). Digestion of each sample collected from six major BBTV-infected banana fields resulted to similar restriction pattern with HaeIII and RsaI while all samples remained undigested with BamHI, EcoRI, EcoRV, HincII, HindIII, PstI and SmaI restriction enzymes.

So far two BBTV groups have been recognized worldwide on the basis of nucleotide sequence of DNA component 1. South Pacific group includes isolates from Australia, Brundi, Egypt, Fiji, India, Tonga and Western Samoa while the Asian group comprises of isolates from Philippines, Taiwan, Vietnam, Southeast Asia and China (Karan *et al.*, 1994; Burns *et al.*, 1995; Dale *et al.*, 1998). However, inter and intra biological differences among isolates of both groups are yet to be determined. Digestions of PCR products of DNA component 1 with HaeIII and RsaI resulted in three and four DNA fragments respectively while no digestion was observed with BamHI, EcoRI, EcoRV, HincII, HindIII, PstI and SmaI. These restriction patterns of all Pakistani isolates reflected a single homogenous population of virus in Pakistan. The homogeneity of current virus population in Pakistan may be due to free movement of suckers by farmers, which has aggravated virus spread to an alarming level. Beside this, air current blowing from Thatta to northward carrying black banana aphids to short and long distances during breeding period, disseminated the same virus isolate to disease free areas.

On comparing the restriction pattern of Pakistani isolate with two known virus groups, it was generally found identical to South Pacific group but closely related to Egyptian and very closely related to Australian isolates. However, none of Asian group isolates has this type of restriction pattern. Interestingly, it is a well documented fact that banana suckers were introduced to Pakistan from India, Bangladesh, Australia etc. as earlier reported by Baluch *et al.*, (1991) and Khalid *et al.*, (1993)). It is generally believed that this could be the only possible reason that BBTV was introduced from Australia through sucker importation. Hence, restriction pattern and group similarity of Pakistani isolate with Australian isolate supports this hypothesis. However, sequencing of Pakistani isolate will ravish its exact origin and evolution.

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