TECHNICAL SHEET No. 1

Virus Detection: *Banana bunchy top virus* (BBTV)

Method: Hybridization with radioactive and non-radioactive probes

General

Virus detection: BBTV from banana tissues
General methods: Hybridization methods, both radioactive and non-radioactive probes

Developed by

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Goals

Develop rapid techniques for BBTV diagnosis in banana plants and banana aphids using radioactive and non-radioactive DNA probes.

Introduction

Banana (*Musa spp*.) is one of the world's largest and most important agricultural commodities. This tropical and subtropical crop contributes significantly to many national domestic and export incomes and in some regions is the principal source of carbohydrate. The success of virus disease control is dependent on the availability of accurate, sensitive, low cost and simple diagnostic techniques, which enable the early detection of virus infections in plant materials.

Information on *Banana bunchy top nanovirus* (6 cssDNA components) can be found on the WEB:

General descriptions:

http://life.bio2.edu/ICTVdB/index.htm
http://www.im.ac.cn/vide/descr056.htm
http://www.ictvdb.iacr.ac.uk/Ictv/fs_nanov.htm
http://www2.ctahr.hawaii.edu/oc/freepubs/pdf/PD-12.pdf (extension bulletin with images)

A list of some references:

http://www.bspp.org.uk/mppol/1997/0624karan/refs.htm

Material and Methods

DNA Extraction

DNA of BBTV was extracted from banana tissues using the Dellaporta technique (Dellaporta, et al., 1983) as following:

1. Grind 0.2 g from leaf midrib tissue or disk from infected plants with Kontes pestles in a 1.5 ml microfuge tube with 500 µl of Dellaporta buffer (100 mM Tris pH 8. 50 mM

ethylenediamine-tetraacetate EDTA, 500 mM NaCl, 10 mM beta mercaptoethanol (BME), Fisher Chemicals).

2. Add 33 µl of 20% sodium dodecyl sulfate (SDS, Fisher Chemicals) (w/v) and vortex and incubate the mixture for 10 min at 65°C.
3. Add 160 µl of 5 M potassium acetate KoAc (Sigma chemicals) and vortex.
4. Spin for 10 min at 10,000 rpm in a microfuge using (centrifuge 5415C).
5. Transfer 450 µl of supernatant to a new tube, avoiding the tissue debris.
6. Add 450 µl phenol, chloroform and isoamyl-alcohol (PCI) 25:24:1 and vortex for 5 min and then spin for 5 min at 10,000 rpm. Remove 400 µl of the upper phase to a clean microfuge tube and add 0.5 volumes of isopropanol, vortex and spin for 10 min at 14,000 rpm.
7. Remove the supernatant, the total nucleic acid was precipitated in the bottom of the tube.
8. Wash the pellet with 70% ethanol and spun 5 min at 10,000 rpm.
9. Resuspend the pellet in 200 µl of TE-RNase buffer (Tris EDTA-RNase buffer).

Designing the primers

Specific primers (A. Rezk and M.K. Nakhla, Univ. of Wisconsin-Madison) were designed to detect the presence of BBTV in infected banana tissues. for each component using Australian isolate sequence from Genbank no. L41574 to L41578. The primers, bbtv1v763, and bbtv1c768, were used to amplify component 1. The primer bbtv2v775 was designed as a general primer for components 2, 3, 5 and 6, and bbtv2c617, bbtv3c703, bbtv5c625 and bbtv6c549 were used as specific primers for components 2, 3, 5, and 6, respectively. The primers bbtv4v527 and bbtv4c526 were specific for component 4.

Table 1. Primers used in the PCR amplification of the six components of the isolated BBTV.

Comp. No.	Primer name	Sequences of primers	Expected size
		5----------3	fragment
BBTV 1	bbtv1v763	AATTGTAGACTGTATAATTACGAGG	1111 bp
	bbtv1c768	AAATGTCTACATATATCCAATGATTTTCC	
BBTV 2	bbtv2v775	TACAAGACGCTATGACAAATGTACKGG	929 bp
	bbtv2c617	CTAAACACCTGTTAATCATGCTTCGCC	
BBTV 3	bbtv3c703	CTTACTCCAGAACTACAATAGAATGCC	973 bp
BBTV 4	bbtv4v526	AGATCAAGAACCGGCTGTGATACC	1050 bp
	bbtv4c525	CGTCTGTCTTCCACAATACCTCTGCC	
BBTV 5	bbtv5c625	ACTCCTACATCTTCTTCCTCTGTCG	801 bp
BBTV 6	bbtv6c549	CCGAATGGTACTATGAGTACTGGACGC	813 bp

Polymerase Chain Reaction (PCR) detection:

PCR Mixture

1. The PCR mixture contains 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 2.5 mM $MgCl_2$; 2.5 mM each of dNTPs, 10 pmoles each primer; 1.0 unit *Taq* DNA polymerase (Promega), and dH_2O to a final volume of 45 µl.
2. Add 5 µl extracted DNA from infected plant tissue to the mixture.

3. Cover the reaction mixtures with 50 µl of mineral oil and then subject to one cycle for denaturation at 94°C for 3 min, 30 cycles of 94°C for 1 min, 55°C for 2 min for annealing, 72°C for 2 min for extension, and finally 1 cycle at 72°C for 4 min, decrease the temperature then to 4°C until the reaction mixtures remove from thermocylcer.

Electrophoreses analysis

The PCR products in 0.7% agarose gel in 0.5% TBE buffer (Tris-Borate-EDTA electrophoresis buffer, 90 mM Tris acetate, 90 mM boric acid and 2 mM EDTA) and stained with ethidium bromide (10 µl/ml) (Sambrook et al., 1989). Visualize the bands by examination under a UV trans-illuminator and photograph using a (MP4 Polaroid camera).

DNA hybridization

A: preparation of the probes

(A-1): Preparation of digoxigenin-labeled BBTV-DNA probe

Incorporate Digoxigenin-11-dUTP (Dig-11-dUTP), (Boehringer Mannheim, Indianapolis, IN,) into the newly synthesized DNA during 30 cycles of PCR with melting, annealing and extension conditions of 1 min at 90°C, 2 min at 55°C and 2 min at 72°C and one cycle 5 min at 72°C (Saiki et al., 1988).

For each reaction a final concentrations of:

0.2 µM of each primer,
10 mM dATP, dCTP, dGTP; 0.65 mM dTTP;
0.35 mM Dig-11-dUTP and
1.2 U *Taq* DNA polymerase (Promega Corporation)
in 50 µl containing 200 ng target DNA.

Confirm the presence of the expected molecular weight PCR amplified product by agarose gel electrophoresis (Sambrook et al., 1989).

(A-2): Preparation of Biotin-Labeled Probes (Chemiluminescent Detection System)

1. Dissolve 100 ng from DNA-PCR product in 5-20 µl of distilled water in a microcentrifuge tube on ice.
2. Add 20 µl 2.5X Random Primers Solution (125 mM Tris-HCl pH 6.8, 12.5 mM $MgCl_{2,}$ 25 mM 2-mercaptoethanol, 750 µg/ml oligodeoxyribonucleotide primers, random octamers) and denature by heating for 5 min in boiling water bath then immediately cool on ice.
3. Add 5 µl of 10X dNTP Mixture (1 mM biotin-14-dCTP, 1 mM dCTP, 2 mM dATP, 2 mM dGTP, 2 mM dTTP in 10 mM Tris-HCl pH 7.5, 1 mM Na_2EDTA) and add distilled water to a total volume of 48 µl and mix briefly.
4. Add 2 µl of Klenow Fragment (40 units/µl Klenow Fragment in 50 mM Potassium Phosphate pH 7.0, 100 mM KCl, 1 mM DTT and 50 % Glycerol) to the reactions and mix gently but thoroughly.
5. Centrifug for 15 – 30 sec and incubat at 37 °C for 60 min to over night in water bath and then add 5 µl Stop Buffer (0.5 M Na_2EDTA, pH 8.0).

Separate unincorporated nucleotides from biotinylated DNA probe by either of two methods:

(I)- Repeated ethanol precipitation:

1. Add sodium acetate 3M, 1/10 volume and two volumes cold ethanol absolute to the reaction tube and mix by inverting the tube.
2. Incubate the tube at -70 °C for 15 min and centrifuge at 15,000 rpm for 10 min.
3. Remove the supernatant carefully with a pipette, dry the pellet and resuspend in 50 µl distilled water.
4. Precipitate the probe again with sodium acetate and ethanol as described above.
5. Resuspend the probe in TE buffer (10 mM Tris-HCl pH 7.5 and Na_2EDTA) and store at –20°C till used.

(II)- Column chromatography:

Use one-ml column of Sephadex™ (Pharmacia LKB Biotechnology) with TE buffer (Feinberg and Vogelstein, 1983; Feinberg and Vogelstein, 1984).

(A-3): Preparation of ^{32}P-labeled BBTV-DNA probe:

For the specific DNA probe, amplify the BBTV-DNA by PCR then ^{32}P labeled using Prime-a-Gene labeling system (Promega Corp.), which incorporated ^{32}P dCTP by random priming.

1. Denature DNA templates by boiling in a water bath (100°C) for 10 min.
2. Add the following to 50 ng of denatured DNA:
   1 µl each of dATP, dTTP, and dGTP,
   2 µl reaction mixture, and
   5 µl ^{32}P dCTP, adjusted the volume to 19 µl with water and add 1 µl of sequenase (Sequenase version 2.0 T7 DNA polymerase).
3. Incubate the reaction at 37°C for 30 min.
4. To stop the reaction, add 45 µl TE (10 mM Tris. HCl, 1 mM EDTA, pH 8.0 and 5 µl dye (4% dextran blue, 0.2% orange G).
5. Remove the unincorporated nucleotides using sephacryl-300 Spin column.
6. Pipette the probe into the middle of the column (75 µl) and centrifuge for 10 sec at 4,000 rpm in a 1.5-ml microfuge centrifuge, after which the probe is recovered from the bottom of the column in a microfuge tube (Technical Bulletin, Promega Corp, 1997).

B: Dot blot, squash blot and tissue blot hybridization using biotin-labeled Probe:

(B-1): Dot blot:

Extract the total nucleic acid from infected healthy banana tissues (frozen tissues or dry tissues) as described above and resuspend the pellets in 50 µl TE buffer. Dilute the extracted DNA and PCR product as follows:

DNA Dilution	1 normal	½,	¼	1/8,	1/16	1/32	1/64	1/128	1/256	1/512…
PCR product dilution	10 ng/µl	1 ng/µl	100 pg/µl	10pg/µl	1 pg/µl	1/10 pg/µl				

Spot 5 µl of the extraction and different dilutions of DNA onto Hybond N+ nylon membrane (Qiabrane Nylon Plus, Qiagen). Allow the membrane to air dry before lysing.

(B-2): Squash blot and Tissue blot:

1. Excise disks (1 centimeter in diameter) from infected and healthy banana leaf tissues using a sterilized cork borer.
2. Place the leaf disks on Qiabrane Nylon Plus Membrane and crush the tissues on the membrane using a sterilized Kontes Pestles.
3. For Tissue Blot, cut the pseudostem or midrib of a small plant using a sterilized cutter and dot onto Qiabrane Nylon Plus Membrane.
4. Allow the membrane to air dry before lysing.

(B-3): Southern blot hybridization:

Southern blot hybridization was used to confirm that PCR-amplified fragments were BBTV using the non-radioactive (Biotin- Labeled Probes-Chemiluminescent and Dig-11-UTP) and radioactive labeled ^{32}P BBTV-DNA probe.

1. Run the samples on 0.7% agarose gel with loading dye and visualize using ethidium bromide and visualize the bands by examination under UV trans-illuminator and photograph using a (MP4 Polaroid camera).
2. Rinse the gel in distilled water with shaking to remove the loading dye and eithidium bromide.
3. Denature the DNA in the gel by shaking in 500 ml denaturation buffer (0.5 M NaOH and 1.5 M NaCl) for 10 minutes. At this point soak the membrane in distilled water for 5 min and then in denaturation buffer for 5 min.
4. Spread the saran wrap on top of a Pyrexglass plate, then transfer the denatured gel onto saran wrap and then the wetted membrane over the gel (avoid creating bubbles).
5. Place three wet 3MM Whatman paper over the membrane and a stack of the brown paper towels over the 3MM Whatman paper.
6. Cover all of these by plastic bag to keep the moisture, place Glass sheet over this set-up and put the weight (approximately 500 gm) on the glass sheet.
7. Blot overnight and remove the stack of paper.
8. Flip the right-side up of the membrane, rinse the membrane in 2X SSC twice for 15 min each time and dry the membrane on 3MM Whatman paper to be ready for hybridization.

Lysing the membranes:

1. Place the membranes in a large tray with 3 layers of Whatman 3MM paper saturated with 0.5 M sodium hydroxide for 5 minutes, for denaturation.
2. Transfer the membranes to 1 M Tris (pH 7.4) for 5 min, then transfer to 2X SSC (300 mM sodium chloride, 30 mM sodium citrate) for 5 min, followed by 95% ethanol for 5 min. All incubations with gentle shaking (50 rpm/min.) at R. T.
3. Allow the membranes to air-dry (Gilbertson et al., 1991) and then use directly for hybridization.

Non-radioactive methods:

Hybridization with Biotin-Labeled BBTV-DNA Probe:

1. Wet the membranes in 0.25 M disodium phosphate, pH 7.2, and prehybridize with Hybridization buffer (0.5 M disodium phosphate, pH 7.2, 50% formamide, 0.5 M EDTA and 7% SDS) for 60 minutes at 48°C in hybridization oven.

2. Denature the biotin-labeled BBTV DNA-1 probe by incubation on boiling water for 5 min and then place in ice for 2 min.
3. Dilute the probe in fresh Hybridization buffer (10-100 ng/ml) and add to membranes (10-100 µl per cm^2)
4. Incubate the membranes overnight at 48°C.
5. Wash the membranes 2 X 5 min at room temperature with 2X SSC, 1%SDS (1 ml/cm^2).
6. Wash 2 X 15 min at 65°C with 0.1X SSC, 1% SDS and wash again 2 X 5 min at room temperature in 1X SSC.
7. Wash the membranes 2 X 5 minutes in Blocking Buffer (0.5 ml/cm^2) and incubate for 10 min in Blocking Buffer (1 ml/cm^2).
8. Dilute the Avidx-AP conjugate 1:5,000 in Blocking Buffer.
9. Add 2 µl of Avidx-AP conjugate in 10 ml Blocking Buffer per 100 cm^2 and incubate for 20 min at room temperature with constant agitation in conjugate solution.
10. Wash the membranes in Blocking Buffer (0.5 ml/cm^2) for 5 min at room temperature, wash three times in Washing Buffer (0.5 ml/cm^2) for 5 min at room temperature and wash finally two times with 1X Assay Buffer (0.25ml/ cm^2) for 2 min at room temperature.
11. Drain the membranes by touching a corner on 3MM Whatman paper and place on Saran Wrap on a flat surface.
12. Pipette a thin layer of CDP-Star substrate solution onto the membranes (3 ml/100 cm^2) and incubate for 5 min at room temperature, and drain the excess CDP-Star solution.
13. Place the membranes in development folder, after removing anti-static sheet and expose to film (X-OMAT™ AR Kodak).

Radioactive method

1. Lyse the membranes as described for the non-radioactive with biotin-labeled method.
2. Prihybridize the membrane in the prehybridization buffer {0.125 M sodium phosphate dibasic salt (Na_2HPO_4), 7% SDS, and 1 mM EDTA, pH 8.0} in the hybridization oven.
3. Denature the probe by adding 0.15 volume of 2 M sodium hydroxide and incubate at room temperature for 30 min.
4. Replace the Prehybridization buffer with 10 ml of hybridization buffer with approximately 25 ng of denatured probe and hybridize the membranes overnight at 68C°.
5. Wash the membranes twice with 100 ml (0.25 M Na_2HPO_4, 1% SDS, and 1 mM EDTA, pH 8.0) and once with 100 ml (sodium phosphate monobasic salt (NaH_2PO_4), 1% SDS, 1 mM EDTA, pH 8.0).
6. Blot dry the membranes and expose at –80C° Kodak OG-1 X-ray film (Sigma Chemical Company, St. Louis, MO) with Lanex screens for 12-48 h.

Results

Total nucleic acids were extracted from BBTV-infected banana tissues using a modified Dellaporta extraction method (Dellaporta *et al*., 1983; and Rojas *et al*., 1993), and the pellets were resuspended in 200 µl of TE RNase buffer and 5 µl (250 ng) of total nucleic acid were added to PCR mixture. The polymerase chain reaction assay was successfully used with specific primers to amplify the six components of the isolated BBTV (Fig. 1).

Fig.1 Electrophoresis analysis of the PCR amplified the six components from banana plants infected with the BBTV using specific primers for each. M=1 kb DNA size marker; lane 1 = 470 bp amplified fragment of BBTV DNA using primer pair cBBTV1 and hBBTV1 for detection, lane 2 = 1.1 kb amplified fragment of BBTV DNA C1, lane 3 = 930 bp amplified fragment of BBTV DNA C2, lane 4 =970 bp amplified fragment of BBTV DNA C3, lane 5 = 1050 bp amplified fragment of BBTV DNA C4, lane 6 = 800 bp amplified fragment of BBTV DNA C5, and lane 7 = 810 bp amplified fragment of BBTV DNA C6.

DNA hybridization methods (squash blot, dot blot and Southern blot) using radioactive and non-radioactive probes were used to detect the presence of BBTV in BBTV-infected banana plant tissues and aphids. Comparison between the sensitivity and the reliabilities of the radioactive (^{32}P labeled DNA probe) and the non-radioactive (biotin labeled probe and digoxigenin labeled probe) were carried out.

The specific ^{32}P-dCTP labeled BBTV-DNA-1 probe was successfully used to detect the presence of BBTV-DNA in infected samples using dot blot hybridization method (Fig. 2-B), using different concentration of Dellaporta extracts from BBTV-infected tissues. The results showed that the sensitivity of the radioactive method was 10 pg of DNA and 1/256 with the dilution of the DNA extract.

Data in Fig.2-A showed that the dot blot of DNA-PCR product and DNA extraction using the Dellaporta method, gave a strong reaction with the specific probe of BBTV-DNA biotin-labeled probe. The results also illustrated that there was no reaction with the healthy plants, which was used as a negative control. The results showed that, the sensitivity of the non-radioactive method was 100 pg of DNA and 1/128 with the dilution of the DNA extract.

A **B**

a b c d e a b c d e

Fig. 2: Quantification of different concentration of total nucleic acid (TNA) extracted from banana tissue to determine the efficiency of the radioactive BBTV-C1 labeled probe with ^{32}P (B) and non-radioactive biotin-labeled probe (A). Lanes from up to down: Dilutions of TNA extraction from banana tissues, 1= original dilution, 2= 1/2, 3= 1/4, 4= 1/8, 5= 1/16, 6= 1/32, 7= 1/64, 8= 1/128, 9= 1/256, 10= 1/512. Lane (c,d) dilutions 1, 1/2, 1/4, 1/512, from TNA was extracted from healthy banana tissue. Lane (e), dilutions from BBTV-DNA component 1 PCR product, 1= 100 ng, 2= 10 ng, 3= 1 ng, 4= 100 pg, 5= 10 pg, 6= 1 pg.

Southern blot hybridization was used to confirm that PCR-amplified fragment was BBTV DNA C1 using radioactive labeled ^{32}P BBTV-DNA probe (Fig. 3-C) and non-radioactive with the biotin-labeled probe (Fig. 3-B). The samples were run on 1.0% agarose gel and the

fragments were transferred to nylon membrane. The results of the southern blot hybridization experiment showed that the reaction was strong with the BBTV-DNA (Fig. 3).

A **B** **C**

Fig. 3. Southern blot hybridization of BBTV-amplified DNA analyzed on 1% agarose gel (Fig 3-A) blotted onto nylon membrane and hybridized with the ^{32}P -dCTP labeled probe (Fig 3-C) and or hybridized with the biotin-labeled probe (Fig 3-B). Lane 1, 3 and 4 showed high levels of hybridization signal from PCR product amplified from infected banana tissue (leaf blade, leaf midrib and pseudostem, respectively) and no reaction in lane 2 where the non-infected banana tissue. On the other hand, lane 5, high levels of hybridization signal from *Eco*RI digestion of the potential recombinant plasmid from the cloning of the PCR fragment for BBTV-DNA component as a positive control.

Fig 4. Dot blot hybridization with specific non-radioactive BBTV-DNA probe labeled with digoxigenin.

A **B**

Fig 5: Squash blot hybridization with specific non-radioactive BBTV-DNA component 1 labeling with biotin (Chemeluminscent method). **(Fig. 5-A)** The membrane before developing, **1**: Infected tissues, **2**: Healthy tissues. **(5-B),** After developing, dark spots with infected tissue squashes and nothing with health tissue squashes (bottom squashes).

Discussion

The Dot blot technique was developed through rapid preparation of plant samples for hybridization based on detection and nucleic acid hybridization with cloned probes labeled with

^{32}P, Chemiluminescent–Biotin, and digoxigenin. In this study, the results showed that the radioactive method was more sensitive than Chemiluminescent–Biotin and digoxigenin methods. The sensitivity of radioactive methods (^{32}P labeled probe) was 10 pg of DNA PCR product and 1/256 with the dilution of the TNA extract, while the sensitivity of non-radioactive methods (Biotin-labeled probes) was 100 pg of DNA PCR product and 1/128 with the dilution of the TNA extract and 1/8 of TNA extract with Digoxigenin-labeled probe. From these results, we demonstrated that the non-radioactive with Biotin-labeled probe was more sensitive than Digoxigenin labeled probe and its sensitivity was almost like that of the radioactive method with ^{32}P -labeled probe, but the radioactive method is more hazardous than other techniques.

The squash blot procedure provides a specific rapid and simple means of using molecular hybridization techniques to detect infected plant tissues and insects. The diagnosis of BBTV in infected tissues and aphids by hybridization was performed directly on tissues squashed onto a nylon membrane where no pretreatment of the samples was necessary. Squash blotting was a tool for rapid, large scale diagnostic of BBTV with radioactive with ^{32}P - labeled probe and non-radioactive with biotin-labeled probe.

Southern blotting is a procedure developed for transferring dsDNA and denatured dsDNA from agarose gel to a nylon membrane or nitrocellulose membrane. The blotted DNA was successfully detected using radioactive with ^{32}P-labeled probe and non-radioactive biotin-labeled probe and the sensitivity was almost the same in both methods.

Table (2): Comparison between the sensitivity of radioactive and non-radioactive methods for rapid detection for BBTV of banana tissues using dot blot hybridization assay and three labeled probe, i.e., ^{32}P, Biotin, and Dig.

TNA dilutions	**Radioactive** ^{32}P labeled probe		**Non-radioactive**
Biotin labeled probe		Dig, labeled probe	
1	++++++	+++++	++++
½	+++++	+++++	+++
1/4	+++++	++++	++
1/8	++++	+++	+
1/16	+++	+++	-
1/32	+++	++	-
1/64	++	++	-
1/128	+	+	-
1/256	+	-	-
1/512	-	-	-

References

Feinberg, A. P. and Vogelstein, B. (1983) Anal Biochem. 132:6.

Feinberg, A. P. and Vogelstein, B. (1984) Anal Biochem. 137:266.

Gilbertson, R. L., Hidayat, S. H., Martinez, R. T., Leong, S. A., Faria, J. C., Morales , F., and Maxwell, D. P. (1991) Differentiation of bean-infecting geminiviruses by nucleic acid hybridization probes and aspects of bean golden mosaic in Brazil. Plant Disease 75: 336-342.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988) Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239: 487-491.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular cloning: A Laboratory Manual. 2nd edn. Cold Spring Harbor Laboratory Press.

Southern, E. M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98: 503