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POSSIBLE BACTERIAL ETIOLOGY OF PAPAYA BUNCHY TOP DISEASE

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ABSTRACT

Samples from 95 papaya plants with symptoms of papaya bunchy top (PBT) were obtained with the aid of local cooperators from 12 countries throughout the American tropics. The samples were examined by polymerase chain reaction (PCR) for the presence of 16S rRNA genes of mycoplasma-like organisms, but none were found. No differences were detectable by PCR between PBT-affected papaya DNA samples and samples from healthy plants. Abnormal fluorescence was consistently observed by epifluorescence microscopy in the region between the xylem and phloem in transverse sections of PBT-affected petioles when stained by 4',6-diamidino-2-phenylindole 2HCl (DAPI) or acridine orange. Bacteria were found in the region by transmission electron microscopy and observed in expressed sap by light microscopy. The bacteria were associated with PBT-affected, but not with healthy, papaya plants. The bacteria measured 0.25–0.35 µm in width and 0.8–1.6 µm in length and had a Gram-negative type cell envelope. All attempts to isolate the bacterium in axenic culture have failed.

INTRODUCTION

Papaya (*Carica papaya* L.) is native to the American tropics but is now cultivated widely throughout the tropical and subtropical areas of the world for both local consumption and export of fruit. and to a lesser extent for production of papain. Papaya bunchy top (PBT) is one of the most economically important diseases of papaya in the American tropics (Davis, 1994). Diagnosis of PBT has relied exclusively on symptomotology and, therefore, has been presumptive. Presently, papaya diseases resembling PBT are known to occur on numerous Caribbean islands from Grand Bahama southward to Trinidad and in Central and South America.

Although a viral etiology was first suspected (Bird and Adsuar, 1952; Cook, 1931), a mycoplasmalike organism (MLO) was later reported to be associated with PBT (Story and Halliwell, 1969). Bodies resembling MLOs were observed by transmission electron microscopy in the phloem of PBT-affected papaya plants, and infected plants treated with tetracycline-type antibiotics exhibited a remission of symptoms.

In an effort to develop more specific means to diagnose PBT, numerous attempts were made to isolate and clone DNA of the putative PBT MLO (M. J. Davis and N. A. Harrison,

unpublished). However, all attempts failed. Recently, Deng and Hiruki (1991) described the application of polymerase chain reaction (PCR) technology to detect the 16S rRNA of MLOs and other Mollicutes. We applied this technology to survey papaya plants with PBT symptoms from different locations in the American tropics for the presence of MLOs but none were detected. These results led us to question the role of a MLO in the etiology of PBT. Further investigation led to the discovery of a fastidious bacterium associated with PBT. The results of this study are reported herein.

METHODS

Papaya petiole samples, mostly from plants with typical PBT symptoms, were collected from commercial and experimental orchards in Puerto Rico. Samples from papaya plants with PBT symptoms were also obtained from different countries throughout the American tropics with the aid of cooperators (Table 1). Petioles from plants in Florida where PBT is not known to exist were used as PBT-free controls. Samples from Puerto Rico and Florida were either processed while fresh, stored at -80 °C, or cut into 1–2 cm segments and preserved in an aqueous solution of 5% sodium borate (Sinclair et al., 1992) until use. Petiole samples from other locations were received as segments in 5% borate solution.

Table 1. Origin of petiole samples from papaya plants with foliar symptoms of bunchy top disease of which all tested negative by PCR for mycoplasmalike organisms and the number of samples testing positive by epifluorescence microscopy for fluorescence associated with the disease.

Country	No. of samples	No. with disease-associated fluorescence	Contributor
Antigua	7	1	N. Roberts-Samuel
Barbados	4	2	M. Phillips
Belize	4	3	J. Link
Costa Rica	5	5	F. Elango
Dominican Republic	4	3	J. Borbon
Dominica	4	3	U. Martin
Ecuador	4	0	R. McMillan, Jr.
Grenada	4	2	C. Persad
Puerto Rico	44	42	(this study)
Saint Lucia	4	3	Henry
Saint Lucia	3	3	E. Ambrose
Saint Vincent	4	2	F. Gynsam
Venezuela	4	1	F. Leal
Total	95	70	

A strain of the eastern aster yellows MLO provided by L. N. Chiykowski was maintained in periwinkle (Catharanthus roseus (L.) G. Don). MLO-infected and healthy periwinkle were grown in a greenhouse.

Total nucleic acids were extracted from healthy and infected plant material using a method modified from that of Saghai-Maroof et al. (1984). DNA from periwinkle infected with the MLOs causing eastern aster yellows, western aster yellows and western-X disease, and from coconut palm infected with the MLO causing palm lethal yellowing were provided by N. A. Harrison. DNA concentrations in solution were measured by spectroscopy, and aliquots of the solutions were diluted to a working concentration of 25 ng/µl and stored at 4 °C.

The P1 and P6 primers of Deng and Hiruki (1991) were used. These primers enable PCR amplification of an approximately 1,500bp DNA fragment within the 16S rRNA gene of all Mollicutes. For all PCR amplification experiments, one or more DNA extracts from plants infected with known MLOs were used as positive controls. Distilled deionized water was used as a negative control.

All PCR was performed in a Coy ThermoCycler II Model 110s with the in sample probe. A standard 50 µl reaction mixture (20 mM TRIS pH 8.3, 1.5 mM $MgCl_2$, 25 mM KCl, 0.01% DIFCO Gelatin, 0.05% Tween 20; 1 ng/µl each of primer and template DNA) in 0.6-mL tubes was used for all amplifications. Each reaction mixture was overlaid with one drop of mineral oil. The temperature cycling schedule was 90 sec predenaturation at 95 °C, followed by 35 cycles of: 30 sec at 94 °C, 50 sec at 58 °C, 80 sec for extension at 72 °C. The reaction products were held at 4 °C after cycling.

Amplification products were separated by horizontal agarose gel electrophoresis. Fifteen to twenty microlitres of reaction mix combined with 1/6 volume of tracking dye/loading buffer were loaded per well of a 1% agarose gel, and electrophoresis was carried out in TAE buffer (40 mM TRIS-acetate, 1 mM EDTA, pH 7.8). Afterwards, the gel was stained with 0.5 µg/mL of aqueous ethidium bromide for 10–15 min, washed in water for 30–60 min and then photographed with UV illumination through UV blocking filters. Hind III/ EcoR I digested lambda DNA fragments were used as molecular weight markers.

Free-hand cross-sections (50–100 µm thick) were cut from petiole segments of papaya preserved in borate solution. Sections were flooded with an aqueous solution of DAPI (4',6-diamidino-2-phenylindole 2HCl; Sigma Chemical Co., St. Louis, MO) at 0.4 µg/mL: (Sinclair et al., 1989) for 30–45 min or acridine orange (Fisher Scientific, Fair Lawn, NJ) at 0.2 mg/ Ll (Davis, 1985) for 3 min, rinsed with water, and mounted on microscope slides in 50% aqueous Karo white corn syrup. Sections were examined with an Olympus BHA microscope fitted with a BH-RFL epifluorescence illuminator (100-watt mercury lamp). For sections stained with DAPI, a UG-1 excitor filter (ultraviolet light; peak transmission at 360nm), DM-400 + L-410 dichroic mirror, and a L-435 barrier filter were used. For acridine orange stained sections, a BG-12 excitor filter (blue light; peak transmission at 420 nm), DM-500 + O-515 dichroic mirror, and O-530 barrier filter were used.

Sap was expressed with pliers from freshly cut transverse surfaces near the base of petioles, mixed with an equal volume of acridine orange or DAPI staining solution, incubated in the dark for 3 min for acridine orange or 30 min for DAPI, and examined as wet mounts by epifluorescence microscopy at 1,000x magnification.

Papaya petioles from two plants in Puerto Rico and one plant in Costa Rica with typical symptoms of PBT and from one healthy plant in Florida were examined by transmission electron microscopy. Samples were processed and examined essentially as described by Norris and McCoy (1983).

Petioles from symptomatic leaves were surface disinfested by dipping in 95% ethanol, submerging in 0.5% sodium hypochlorite for 5 min, and rinsing three times with sterile deionized water. The petioles were then homogenized in a sterile blender jar for 30 sec with 4 volumes (w/v) of filter-sterilized (0.2 μm-pore size) 0.01 M potassium phosphate buffer, pH 6.9, containing 5% Trypticase peptone (BBL). The homogenate was filtered through four layers of cheesecloth and centrifuged for 10 min at 10,000 x g. The pellet was resuspended in 10 mL fresh extraction buffer. Serial dilutions in 0.01 M phosphate buffer, pH 6.8, were inoculated onto different culture media. Numerous media were used including agar-solidified preparations and various modifications of the PD2 medium (Davis et al., 1980b), SC medium (Davis et al., 1980a), PW medium (Davis et al., 1981), L20 medium (Davis et al., 1983), and C3G medium (Liao and Chen, 1977). Inoculated media were incubated aerobically at 28 °C and examined periodically for growth for 3–4 wk.

RESULTS

Ninety-five papaya plants with symptoms of PBT from 12 countries throughout the American tropics were assayed by PCR for the presence of 16S rRNA genes of MLOs using the P1 and P6 primer pair, but none were found (Table 1). No differences were detectable between healthy and diseased papaya DNA samples. All positive control amplifications produced appropriately sized amplimers and all negative controls produced no correctly sized amplimers. Low titre and/or interfering substances as a cause of failure to detect MLO DNA in papaya samples were tested by diluting positive indicating MLO DNA extracted with total DNA from periwinkle hosts with thousand- and million-fold relative quantities of papaya DNA extracts. No positive control DNA amplification was diminished by addition of any papaya DNA extracts.

Histochemical differences were observed between petiole sections from healthy papaya plants and those taken from PBT-affected plants. Abnormal fluorescence associated only with PBT-affected tissues was observed on the periphery of the phloem on its innermost side near the xylem and sometimes extended into the phloem rays as far as the cortex. When petiole sections from PBT-affected papaya were stained with DAPI, regions of bright bluish white fluorescence were observed intermixed with a dull yellow autofluorescence. When sections were stained with acridine orange, the same region exhibited a bright yellow

to orange fluorescence. Similar staining was not observed in sections from healthy plants and plants affected by papaya ringspot virus.

When petiole tissues from 148 plants from seven locations in Puerto Rico were examined by epifluorescence, the fluorescence associated with PBT was observed in samples from 65 of 66 (98.5%) symptomatic plants and six of 82 (7.3%) nonsymptomatic plants. Of the 95 samples from 12 countries which tested negative for MLOs in the PCR assay, 70 exhibited the abnormal fluorescence associated with PBT when stained with DAPI or acridine orange (Table 1).

Small bacteria were observed by electron microscopy within papaya petiole cells on the periphery of the phloem. The bacteria were found in all three PBT-affected plants from Puerto Rico and Costa Rica but were not found in a healthy plant from Florida. The bacteria were rod-shaped measuring approximately 0.25–0.35μm in width and 0.8–1.6 μm in length. Their cell wall resembled those of Gram-negative bacteria.

The plant cells in which the bacteria were found appeared to be laticifers. Normal laticifers were not prevalent in the samples from PBT-affected plants but were readily found in the same location within samples from the healthy plant. Cells containing unidentified bodies similar to those presumed to be MLOs in an earlier study (Webb and Davis, 1987) were observed in samples from the two PBT-affected plants from Puerto Rico but not in the similar sample from Costa Rica and the healthy sample from Florida. The bodies did not appear to be bound on the outside by a unit membrane similar to the cytoplasmic membrane of MLOs. The bodies were observed in cells with thick cell walls which might have been laticifers, but the bodies appeared smaller on the average than normal latex vesicles and had more electron dense materials.

Numerous small, rod-shaped bacteria, often greater than 10 per microscope field (1000x), were observed in expressed sap from fresh papaya petioles from 15 of 19 (78.9%) plants with PBT symptoms, including both foliar symptoms and a reduction of latex-flow upon wounding, but were not observed in similar preparations from 13 of 15 (86.7%) plants without PBT symptoms.

All attempts to isolate the bacteria in axenic culture were unsuccessful. Undiluted inoculum preparations sometimes contained as many as 50–100 bacterial cells/microscope field, but only low numbers of fast growing bacterial contaminants were isolated from the preparations.

DISCUSSION

PCR has proven to be a highly sensitive technique for the detection of MLOs and other Mollicutes (Deng and Hiruki, 1991; Ahrens and Seemuller, 1992; Harrison et al., 1994). Constant failure in the present study to detect MLO DNA in papaya with PBT symptoms by PCR indicates that a MLO is not associated with PBT. PCR inhibitors were apparently not responsible for the failure to detect the putative PBT MLO because total DNA extracts from

diseased papaya did not interfere with detection of MLO DNA. The possibility that a MLO is responsible for PBT symptoms in some geographic locations in the American tropics but not in others seems remote because the putative PBT MLO was not detected by PCR in samples from 12 different countries, including four samples from the Dominican Republic where MLOs were first reported as being associated with PBT (Story and Halliwell, 1969).

The two previous reports by Story and Halliwell (1969) and Webb and Davis (1987) associating MLOs with PBT appear to have been erroneous. These reports were based primarily on observations by transmission electron microscopy of bodies resembling MLOs in the phloem of papaya with PBT. The bodies in the electron micrograph presented by Story and Halliwell (1969) could alternatively be interpreted as being degenerate mitochondria. No micrographs showing MLOs were published in the brief note by Webb and Davis (1987); however, 100 micrographs taken during that study were available and examined again. Bodies which had been previously identified as MLOs were present in the majority of the micrographs and were identical to the unidentified bodies observed in tissue from PBT-affected papaya during the present study. No other bodies resembling MLOs were observed. A relationship between PBT and these unidentified bodies has not been determined; however, similar bodies were not observed in sections from healthy plants in either study. One possible explanation for this is that the bodies were latex vesicles which had been adversely affected during the disease process. Collapse of latex ducts due to bacterial infection might be responsible for the cessation of latex-flow observed in the PBT-affected plants.

A fastidious bacterium appears to be associated with PBT. Both light and electron microscope examinations supported this association. Furthermore, the bacteria were found in plant cells by electron microscopy which appeared to be laticifers and were located in the same area as the PBT-associated fluorescence detected by epifluorescence microscopy. This fluorescence was consistently associated with both foliar symptoms of PBT and the reduction or complete absence of latex flow upon wounding. The remission of PBT symptoms after application of tetracycline antibiotics still tends to suggest that a prokaryote might be responsible for the disease, even though a MLO etiology now seems unlikely.

Failure to isolate the bacterium on culture media which support the growth of fastidious prokaryotic plant pathogens has precluded attempts to fulfill Koch's postulates as proof of pathogenicity. Further studies are needed to determine the role of bacteria in the etiology of PBT.

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