

## Bacterial Blight of Cassava in Colombia: Etiology

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Supported in part by a fellowship from The Rockefeller Foundation, the International Center for Tropical Agriculture (CIAT), and the Colombian Institute of Agriculture (ICA).

The assistance of Gary Gaard in the electron microscopy work is gratefully acknowledged.

Accepted for publication 19 July 1973.

### ABSTRACT

The bacterial blight of cassava (*Manihot esculenta*) has increased in severity in Colombia during the past 5 yr. Symptoms on susceptible cultivars include leaf spotting, wilting, die-back, and gum exudation on young shoots, and vascular discoloration in mature stems. The bacterium (CBB) penetrates via the stomata or through wounds in epidermal tissues. It invades the vascular tissues of leaves and young shoots, resulting in extensive breakdown of parenchymatous tissues. In highly lignified tissues of old stems or roots, the bacterium remains restricted to the vascular strands. These symptoms are similar to those reportedly induced by *Xanthomonas manihotis*, but the isolates of CBB differ from the former in cell size, motility, production of  $H_2S$ , utilization of nitrate, hydrolysis of starch, and in several serological characteristics.

CBB is a gram-negative, motile, slender rod, with a single polar flagellum. It is aerobic, fast-growing, and forms no pigments on carbohydrate-containing media. It hydrolyzes starch and gelatin, and reduces litmus milk. It produces levan, catalase, arginine dihydrolase, and lipase,

but not  $H_2S$ , indole, urease, tyrosinase, or phenylalanine deaminase. It grows in ordinary media plus NaCl or tetrazolium chloride at a maximum concentration of 2.5 and 0.2%, respectively. It utilizes nitrate and ammonium as sources of nitrogen, and most of the simple sugars as sources of carbon, but acid is not produced; various amino acids and other organic acids are readily utilized.

Isolates of CBB from distinct geographical areas induced similar symptoms on cassava, but belonged to two different serological groups, each separable into two additional groups on the basis of their ability to utilize sucrose, cellobiose, and trehalose as carbon sources. However, these groupings were not correlated with geographical origin of the isolates.

CBB was separated by serological- and phage-typing methods from three species of *Erwinia*, two of *Pseudomonas*, and ten of *Xanthomonas*, including *X. manihotis*. A *Bdellovibrio* sp. caused lysis of CBB specifically and was used to separate CBB from other plant pathogenic bacteria.

Phytopathology 64:74-82

Cassava (*Manihot esculenta* Crantz) is one of the major sources of food in the tropics. The root of most varieties is palatable for human consumption, and fresh or dried roots, as well as leaves, are used as forage for animals.

In Colombia, efforts to multiply high-yielding cultivars of cassava during the past five years have been seriously hampered by the susceptibility of these clones to a bacterial blight which was particularly damaging in 1971. The pathogen causes extensive damage to leaves, petioles and stems, often resulting in complete destruction of the plant.

At least four bacterial diseases of cassava have been described: (i) In 1928, Hansford (19) described a cassava disease from Uganda characterized by leaf spotting and necrosis of the petioles and stems. The pathogen was named *Bacterium cassavae* sp. n. (10, 19), but it was later renamed *Erwinia cassavae* (Hansford) Burkholder (5); (ii) Wiehe and Dowson (39) reported a disease associated with *Xanthomonas cassavae* sp. n. in Nyasaland characterized by leaf spots which were first yellow and circular, then turned brown and became angular and translucent, and were surrounded by a broad yellow halo; (iii) *Pseudomonas solanacearum* E. F. Sm. was identified as the cause of wilting of young cassava plants in Brazil (2); and (iv) *Xanthomonas manihotis* (Arthaud-Berthet) Starr reportedly caused great losses on varieties of *M. esculenta*, *M. utilisissima* Pohl., *M. palmata* Pax, and *M. aipi* Pax in northern

and central Brazil (1,7,10). Symptoms include angular leaf spots which enlarge and cause necrosis and defoliation, wilting of unligified young branches, and gum exudation from affected plant parts (3, 18). Most of the information on this bacterium stems from the early work of Burkholder (10) who isolated the pathogen from infected cuttings sent to him from Brazil.

Symptoms of bacterial blight of cassava in Colombia were similar to those caused by *X. manihotis*, as described above. Because of the confusion regarding the etiology of the various bacterial diseases of cassava, the investigations reported here were initiated to determine the physiological characteristics of the pathogen in Colombia and to compare them with those of *X. manihotis*.

**MATERIALS AND METHODS.** — *Isolation of the pathogen.* — Infected cassava plant parts were collected from plantings in different areas of Colombia, Venezuela, and Brazil (Table 1). To isolate the pathogen from infected tissues, excised portions (0.5 to 1.0 cm<sup>2</sup>) of the water-soaked margins of each leaf spot, or pieces of stem tissue, were placed in a test tube containing 5 ml of sterile distilled water and allowed to stand for 15 min. Tubes were then shaken for 15 s and a loopful of the suspension was streaked on Kelman's TZC medium (25). After 48-hr incubation at 30 C, wild-type colonies were selected on the basis of their color, fluidity, and other

TABLE 1. Geographic origin of CBB (bacterial isolates from blighted cassava)

Colombian collection number	Date of collection	Location	Isolated from
1.23L	8-10-70	Moniquira (Cundinamarca, Col.)	stem <sup>a</sup>
2.24L	9-1-70	Marconia (Magdalena, Col.)	leaf <sup>b</sup>
3.25L	9-5-70	Dorada (Caldas, Col.)	stem, leaf
4.26L	8-5-70	Chicoral (Tolima, Col.)	stem, leaf
5.27L	9-26-70	Fresno (Tolima, Col.)	stem, leaf
6.28L	10-5-70	Sta. Lucia (Bolívar, Col.)	stem, leaf
7.29L	10-12-70	Villavicencio (Meta, Col.)	stem
8.30L	10-20-70	Medellin (Antioquia, Col.)	stem, leaf
9.31L	10-30-70	Monteria (Córdoba, Col.)	stem, leaf
10.32L	12-22-70	Monteria (Córdoba, Col.)	stem, leaf
11.33L	4-30-71	Palmira (Valle, Col.)	leaf
12.34L	5-30-71	Maracaibo (Venezuela)	stem
13.35L	5-30-71	São Paulo (Brazil)	stem
14.36L	5-30-71	São Paulo (Brazil)	culture <sup>c</sup>
15.37L	8-30-71	Mariquita (Tolima, Col.)	leaf

<sup>a</sup>Stem with dieback symptoms.

<sup>b</sup>Leaf with necrotic spots.

<sup>c</sup>Culture received from L. A. Pereira, Instituto Biológico, São Paulo, Brazil

characteristic features. Representative isolates of each geographical area were stored at room temperature in capped test tubes, each containing a suspension of the bacterium in 10 ml sterile distilled water.

*Inoculation procedures.* — Four different methods were used to inoculate 2-mo-old cassava plants (cultivar Llanera) grown from stem cuttings planted in sterilized sandy soil in pots:

— 1) Spray inoculation. — A bacterial suspension containing  $1-3 \times 10^9$  cells/ml sterile distilled water was mixed with either Carborundum (0.1 g/liter), agar (0.2%), gelatin (0.2%), dextrose (0.2%), or Tween 20 (polyoxyethylene sorbitan monoleate) (0.01%). Five plants were each inoculated with one of the above suspensions by means of a DeVilbiss atomizer connected to an air pump at  $7.03 \times 10^{-3}$  kg-force/mm<sup>2</sup> (10 lb/in<sup>2</sup>) pressure. There were three replications of each treatment. Plants were incubated for 20 days in a growth chamber at 30 C, 90-100% relative humidity, and 12-hr photoperiod 19,380 - 21,500 lx (1,800 to 2,000 ft-c) provided by Sylvania Gro-Lux and General Electric cool-white fluorescent lamps and incandescent bulbs.

— 2) Leaf-rubbing. — Twenty leaves from five plants were each rubbed with cheesecloth or bare fingertips which had been moistened with a bacterial suspension of  $3 \times 10^9$  cells/ml. Inoculated plants were incubated in a growth chamber as above.

— 3) Stem-puncture. — Ten plants were inoculated at the third and fourth leaf axils from the apex by forcing a sharp needle through a drop of a bacterial suspension ( $3 \times 10^9$  cells/ml) and into the stem. Plants were maintained in the greenhouse at 25 C ( $\pm$  8 C) and 50-80% relative humidity.

— 4) Root inoculation. — Roots of 20 plants were cut with a knife and 50 ml of a bacterial suspension ( $3 \times 10^9$  cells/ml) were then poured on the soil of

each pot. The procedure was repeated after a 15-day interval. Plants were grown in the greenhouse as above.

*Histological methods.* — Small segments (1 cm<sup>2</sup>) from spray-inoculated leaves were removed every 10 hr up to 60 hr and at 4, 6, and 8 days after inoculation. These samples were fixed with formaldehyde-acetic acid-ethanol (FAA) solution and dehydrated by the N-butyl alcohol method (23), embedded in paraffin, and sectioned with a microtome. Leaf sections (10-15  $\mu$ ) were stained by the iron-alum-hematoxylin method (23).

*Characterization of the causal agent.* — The CBB bacterium may be identified on the following bases:

— 1) Morphology. — The morphology of individual cells and colonies of the pathogen was determined from single-cell cultures of isolates 1.23L and 9.31L. Preliminary observations on motility were made on hanging drops prepared from a 24- or 48-hr-old liquid culture on TZC basal medium (TZC minus tetrazolium salts and agar) of either isolate.

Cells from 24-, 48-, and 72-hr-old TZC liquid cultures were stained by the Hucker modified gram stain procedure (33). Observations of flagellation were made on cells from 18-hr-old TZC liquid cultures prepared according to the Leifson (33) and Blair et al. (6) methods. Electron microscope preparations were made from 24- and 72-hr-old TZC agar cultures by the negative-staining method and by shadow casting (8, 22). For shadow casting, a dilute suspension ( $5 \times 10^7$  cells/ml) of bacteria (isolate 9.31L) in 10 ml Kelman and Hruschka's motility medium (26) was incubated for 4 hr at 30 C. This suspension was then diluted 1:2, 1:3, and 1:4 with 0.5% bovine serum and a droplet of each suspension was placed on a carbon-coated nitrocellulose grid and allowed to dry. Bacterial specimens were coated with

vaporized platinum and examined with the electron microscope.

– 2) Growth rate. – The growth curve of isolate 9.31L at different temp was determined in 250-ml Erlenmeyer flasks, each containing 50 ml of TZC liquid medium and provided with a colorimeter tube fused to the side. Flasks were shaken in a water bath and the OD<sub>600 nm</sub> was determined every 2 to 3 hr with a Bausch and Lomb Spectronic 20 colorimeter.

– 3) Biochemical and physiological characteristics. – Standard bacteriological tests for CBB, including reduction of litmus milk, levan production, salt tolerance, and production of various enzymes, were performed according to the methods cited in Table 2.

Tests for ability to rot potato, carrot, and cassava roots were carried out as follows: washed, peeled, surface-sterilized tubers or roots were cut transversely into small disks, 7- to 10-mm thick. Each slice was placed in a petri dish and 5 ml of sterile water was added. A loopful of bacteria from a 48-hr-old culture of each isolate was added to the center of each slice. The test was considered positive if the slice was completely rotted 3 days after inoculation (31).

Induction of the hypersensitive reaction was determined on leaves of 1-month-old tobacco (*Nicotiana tabacum* L. 'Bottom Special') plants. Leaves were infiltrated with suspensions of  $1-5 \times 10^9$  cells/ml of 14 isolates of CBB. Each isolate was infiltrated into an area 3 to 5 cm<sup>2</sup> in each of 10 to 12 areas between the principal lateral veins with a hypodermic syringe fitted with a fine (No. 30) needle (28).

The replica plating technique (29) was used to test isolates of CBB for their ability to grow on different carbon and nitrogen sources. Fourteen isolates of CBB were grown for 24 hr as separate colonies on a single nutrient-agar master plate which was then used for replica plating the test media. These media were prepared by adding the carbon or nitrogen source to the standard mineral base of Misaghi and Grogan (31). Sources of carbon were added at 0.1% (noncarbohydrates) or 0.2% (carbohydrates) (w/v) to the mineral base containing 0.1% (w/v) ammonium sulfate. Nitrogenous inorganic substrates (0.1%) were added to the standard mineral base containing 0.2% glucose (31). Plates were incubated at 30 C and growth evaluations were made after 1 wk.

The sensitivity of the 14 isolates of CBB to antibiotics was determined by means of Bacto-sensitivity disks (Difco Laboratories, Detroit, Mich.) following standard procedures recommended by the manufacturer.

*Serological methods.* – Bacteria were grown on NA for 48 hr at 28 C and cells were collected, washed by centrifugation, and suspended in 0.85% saline at  $1 \times 10^9$  cells/ml. This suspension was injected into the marginal ear veins of rabbits. Each rabbit was given a series of five daily injections (1 ml each) followed by a 5-day rest period before the sixth, and final, injection. Agglutination tests were made to determine the optimum bleeding period. Antiserum was preserved with 0.01% merthiolate and maintained in

the refrigerator (4 C) or frozen (–10C).

A dilution series of antiserum ranging from 1:16 to 1:1,204 was prepared. One milliliter of a bacterial suspension ( $1.5 \times 10^8$  or  $4.5 \times 10^7$  cells/ml) in saline was added to 1 ml of each serum dilution. Tubes were shaken and incubated at 30 C. Agglutination was recorded 1 hr later.

For double-diffusion tests, each bacterial strain or species was grown on TZC liquid medium for 24 hr. The cultures were centrifuged at 365 g for 1 hr and the pellets were suspended in 2.0 ml saline to serve as the antigen. Antiserum (diluted 1:16) was added to the central well of an Ouchterlony double-diffusion plate and bacterial antigens were added to the surrounding wells. Each antiserum-antigen combination was replicated at least five times. The dishes were incubated at 30 C and high relative humidity for 5 days.

*Bacteriophage and Bdellovibrio sp. typing.* – Bacteriophage and *Bdellovibrio* sp. capable of lysing CBB cells were isolated from soil samples collected in an old, badly infected planting of cassava at Espinal (Tolima), Colombia. Ten g of soil were suspended in 100 ml of sterile distilled water and stirred for 15 min. After soil particles had settled, the supernatant fluid was centrifuged for 1 hr at 1465 g. The clear fluid was filtered through a Millipore filter (0.45 $\mu$ ) and 1 ml of this solution was used to inoculate 9 ml of a suspension ( $1 \times 10^9$  cells/ml) of each bacterial isolate. Five ml of this suspension were added to 100 ml of melted (45 C) NA medium which was then poured into sterile petri dishes. After 2 to 4 days of incubation in a growth chamber at 28 C, isolated plaques of either bacteriophage or *Bdellovibrio* sp. were used to inoculate either strains of CBB or species of other plant pathogenic bacteria as follows: Fifty ml of a heavy suspension of the infected bacterial host were centrifuged at 1,465 g and the supernatant fluid was filtered through a Millipore filter (0.45 $\mu$ ). One ml of this solution was used to inoculate 9 ml of a suspension ( $1 \times 10^9$  cells/ml) of the test bacterium. This suspension was then diluted to  $10^{-2}$  or  $10^{-4}$ . One ml of each diluted suspension was added to 100 ml of melted (45 C) nutrient agar. Inoculated medium was then poured in petri dishes, allowed to solidify, incubated for 7 days at 30 C, and then checked visually for the presence of lysed areas. Isolates which showed no lysis were tested three additional times by the same procedure.

**RESULTS.** – *Symptomatology.* – Initial symptoms of the disease appeared as water-soaked angular areas, clearly distinguishable on the abaxial surface of the leaves. These lesions became brown or dark-brown and, in some cultivars, a yellow halo surrounding the spots appeared. Spots enlarged and coalesced, forming a large necrotic area (Fig. 1). Necrotic areas sometimes extended throughout the entire lobe. Leaf spots often exuded a yellowish, sticky material that collected in droplets, mostly on the lower leaf surface and along the veins or veinlets. Veins became discolored, and this discoloration extended away from the margins of the spots. As the disease progressed, petioles turned brown to

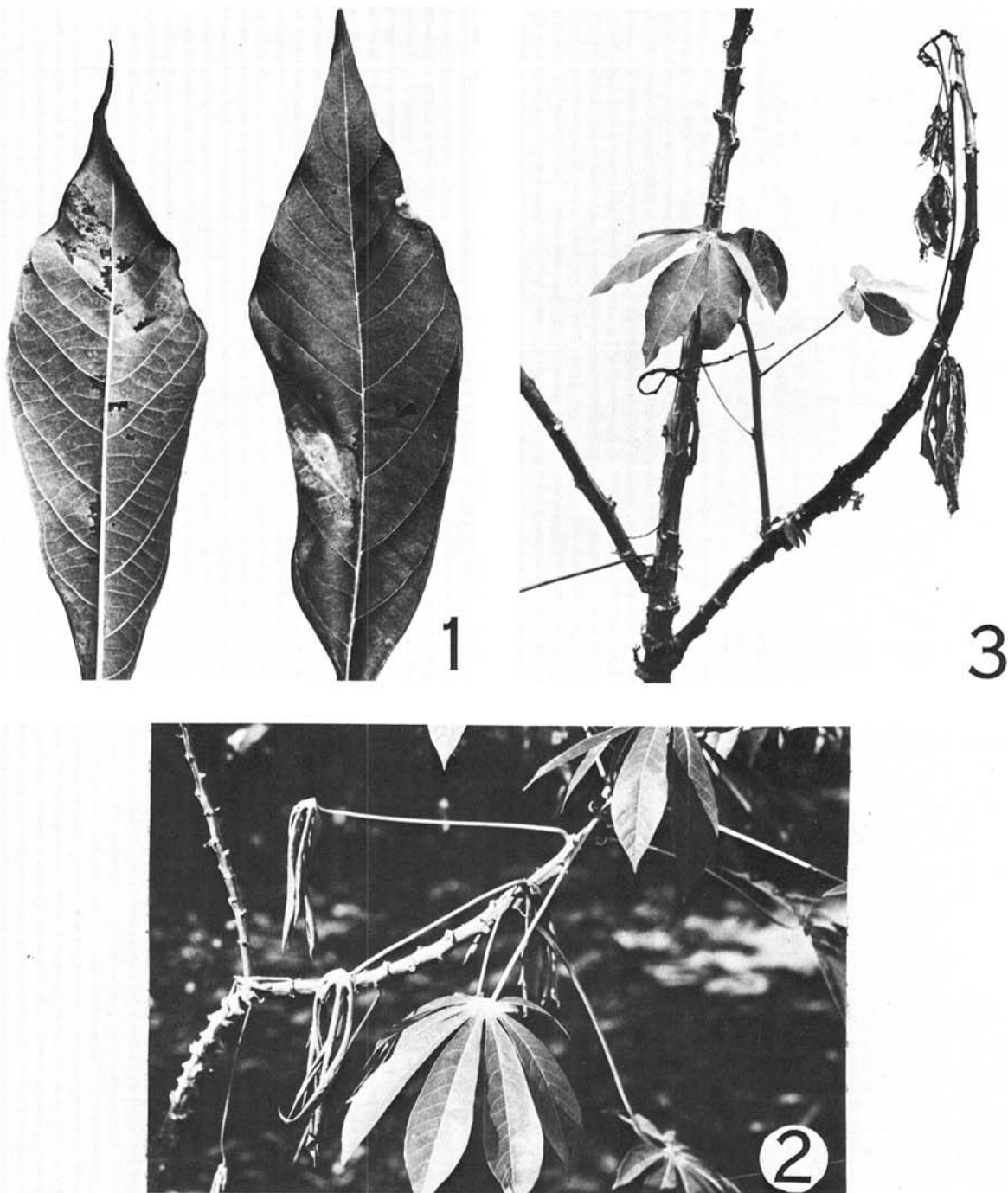


Fig. 1-3. 1) Cassava leaf lobes showing brown and dark-brown leaf spots which have enlarged and coalesced, forming a large necrotic area on the leaf lamina. 2) Infected cassava plant showing wilted, necrosed leaves still attached to the stem. Note that young or emerging leaves are not affected by CBB. 3) Sprouting cassava plant after the top was heavily infected by CBB. The new shoots that emerged apparently remained healthy during the rainy season.

dark-brown and the entire leaf wilted. Wilted leaves remained attached to the stem, but abscised once they became dried. Only fully expanded leaves were infected; young or emerging leaves were not infected

(Fig. 2). The epidermis of infected, young, green stems, or of petioles, often developed cracks through which drops of a bacterial exudate oozed out and dried as a yellowish, glistening scab. Infected young



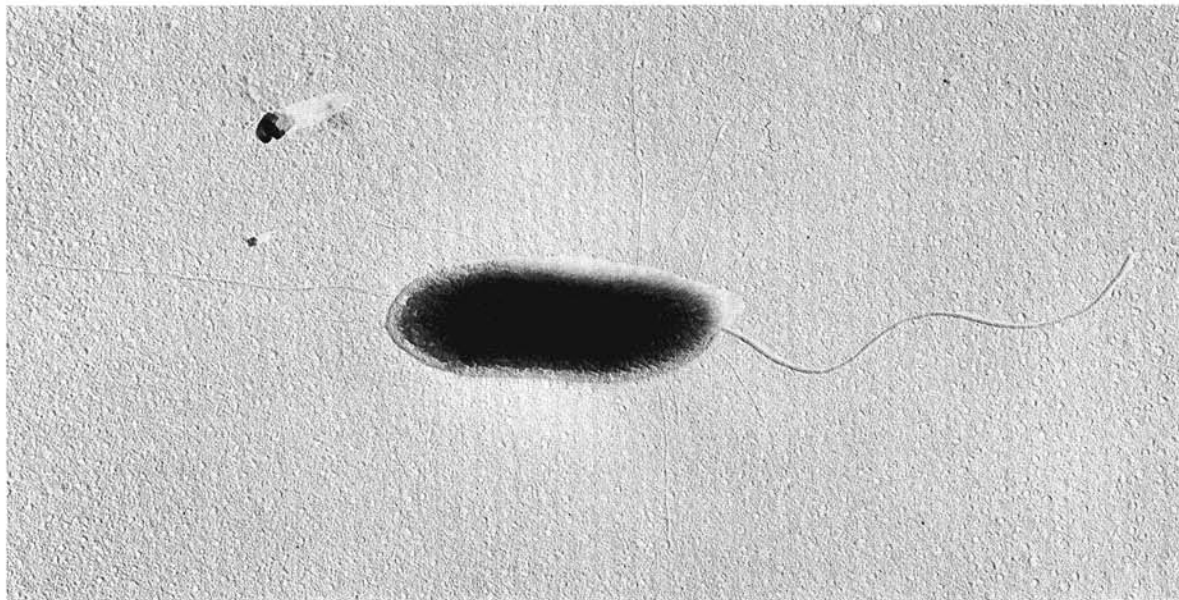


Fig. 4. Electron photomicrograph (X 32,640) of a cell of CBB, isolate 9.31L. Image of a freeze-dried bacterium coated with vaporized platinum under vacuum. Photograph by Mr. G. Gaard.

stem tissues rotted, particularly in areas surrounding primary infection sites, resulting in a characteristic die-back symptom. Cross sections of infected young stems often revealed light-brown to black vascular strands, but mature stems did not appear to be invaded in most cultivars.

When severe infections killed the top of a plant, sprouting occurred at the base of the stem (Fig. 3). If these sprouts were infected and killed, additional sprouting did not occur. Generally, sprouts developing from infected cuttings became diseased and died within a few days after emergence.

Roots of infected plants usually remained healthy, except when infection occurred on young, susceptible cultivars, such as 'M. Col. 1'. In this case, roots showed dry, necrotic vascular strands, but rotting was restricted to the vascular tissues.

*Cultural characteristics.* — On TZC medium, after 48 hr incubation at 30 C, CBB developed fluidal, round colonies which were shiny, convex, entire, with a reddish center surrounded by a pale, bluish halo. The morphology of colonies from cultures originating from single cells was similar to that of isolates obtained directly from diseased plant tissues. Isolates obtained from different geographical areas of Colombia, Venezuela, and Brazil (Table 1) showed identical colony characteristics.

Plants which were sprayed with CBB cell suspensions showed water-soaked angular spots on infected leaf lobes 8-10 days after inoculation. Three days later, tiny, milky drops exuded from the spots. By 20 days after inoculation, spots had enlarged and coalesced, covering part of or the entire lobe lamina. Similar symptoms were obtained on plants inoculated

by the leaf-rubbing method. Of the various inoculum additives tested, only Tween 20 increased infectivity (65% over control).

In plants inoculated by the stem-puncture method, wilting of leaves appeared near the point of inoculation 5-6 days after inoculation. Afterwards, additional leaves wilted, the stem exuded gum profusely, and, by 20 days after inoculation, the entire plant had wilted.

With one exception, all root-inoculated plants showed no noticeable symptoms of the disease, even after 4 months. The pathogen could not be reisolated from the inoculated root tissues. Discolored, necrotic vascular strands were observed only in the roots of the highly susceptible cassava clone M. Col. 1 4 mo after inoculation.

There were no differences in virulence among isolates, with the possible exception of isolate 5.27L which caused complete wilting of the inoculated plants by 9 days after inoculation, as compared with 15-20 days for most isolates.

*Penetration of the host.* — CBB penetrates the host through the stomata. Masses of bacteria could be found within the stomatal cavities 40 hr after inoculation and, at 60 hr., adjacent parenchyma cells had begun to disintegrate. After 4 days, the pathogen had moved to the protoxylem and protophloem of adjacent veinlets, and cells of the abaxial epidermis and spongy parenchyma tissues had become necrotic. Eight days after inoculation, bacterial cells were observed inside the vascular strands, and cells of the palisade layer and adaxial epidermis were disintegrating.

CBB moved systemically from leaf lesions to the stem via the petioles. The pathogen was able to move

TABLE 2. Biochemical and physiological characteristics of 14 isolates of CBB (bacterial isolates from blighted cassava)

Test	Reaction	Method Lit. Ref. No.
Gelatin hydrolysis and liquefaction	Positive	31,33
Starch hydrolysis	Positive	33
Reduction of litmus milk	Positive	33
Levan production	Positive	31
Oxidase	Negative	6,31
Lipase	Positive	31,35,36
Catalase	Positive	6
Urease	Negative	6
Arginine dihydrolase	Positive	31
Phenylalanine deaminase	Negative	6
Tyrosinase	Negative	6,11
Soft rot of potatoes, carrots, and cassava roots	Negative	31
Hypersensitive reaction in tobacco leaves	Negative	28
H <sub>2</sub> S from peptone and cysteine	Negative	6
Metabolism of glucose	Oxidative	6
Methyl red and Voges-Proskauer reactions	Negative	6,33
Nitrate reduction	Negative	6,31
Nitrite from nitrate	Negative	6,33
NH <sub>4</sub> <sup>+</sup> from nitrate	Negative	6,33
Salt tolerance	Maximum 2.5%	11,35
TZC tolerance	Maximum 0.2%	30
Na-polypectate utilization	Positive	6,11
Indole	Negative	33
Fluorescence on King's medium B	Negative	27
Poly- $\beta$ -hydroxybutyrate	Positive	6

extensively through young stem tissues of the cultivar Llanera, but was unable to invade the mature, basal part of the stem.

*Cell and colony morphology.* — CBB is a slender gram-negative rod. Measurements from electron photomicrographs of 24-hr-old cells, grown in TZC liquid medium, indicate an average size of 1.8  $\mu$  (range: 2.69 to 0.76  $\mu$ ) by 0.39  $\mu$  (range: 0.49 to 0.32  $\mu$ ). The cells bear a single polar flagellum; numerous pilli are present on each cell. Cells are not encapsulated, do not form spores, and are single or form short chains of 3-4 cells (Fig. 4).

The bacterium grows in ordinary culture media, does not produce pigments, but reduces tetrazolium salts. On sucrose-containing media, colonies are very slimy.

*Biochemical and physiological characteristics.* — The mass-doubling time of CBB isolate 1.23L in TZC liquid medium at 32 C was 46 min; at 30 C, 47 min; at 28 C, 55 min; and at 34 C, 57 min. The optimum temp range for growth was 30 to 32 C. The bacterium did not produce visible growth at 4 or 39 C.

All 14 isolates of CBB were similar in most physiological and biochemical characteristics (Table 2). The utilization of a wide variety of organic compounds, following the determinative scheme of Misaghi and Grogan (31) and others for pseudomonads, did not prove diagnostically useful to differentiate strains within the 14 isolates tested, as indicated below:

— 1) Carbohydrates and sugar derivatives. — The bacterium grew well, with weak acid production, on basal medium plus D-mannose, D-glucose,

L(-)fructose, or melibiose but grew poorly, with slight acid production, on basal medium plus L-rhamnose, D(-)ribose, or maltose. The bacterium also grew well on basal medium plus D(+)xylose or L(+)-arabinose, but no acid was produced. The organism grew poorly on basal medium plus D-galactose, D(-)arabinose, fructose, or raffinose; and did not grow with salicin, saccharin, or lactose.

The utilization of sucrose, cellobiose, and trehalose by CBB isolates varied. Two different biochemical groups could be distinguished: (i) isolates 4.26L, 5.27L, 7.29L, and 11.33L grew well on basal medium plus any of these carbohydrates; (ii) the remaining isolates grew poorly with these same carbohydrates as the source of carbon.

— 2) Fatty acids. — None of the isolates was able to utilize sebaceate, octonate, valerate, isovalerate, isobutyrate, linoleate, adipate, suberate, nonanoate, hexadecanoate, dodecanoate, decanoate, or acetate.

— 3) Miscellaneous organic acids. — None of the isolates utilized azelate, fumarate, itaconate, phthalate, benzoate, glutarate, salicylate, ascorbate, pimelate, nicotinate, anthranilate, or D(-)mandelate, but all utilized citrate, polygalacturonate, glutamate, and 2  $\alpha$ -ketoglutarate.

— 4) Amino acids. — The bacterium was able to utilize L-alanine, L- and D-glutamate, L-arginine, DL-threonine, DL-methionine, L-cystine, and DL-cystine. No growth was observed with  $\beta$ -alanine, DL-serine, L-isoleucine, L-lysine, DL-valine, or glycine. Isolates varied in the utilization of DL-alanine, L-leucine, and D- or L-tryptophan, L-proline, OH-L-proline, and L-tyrosine.

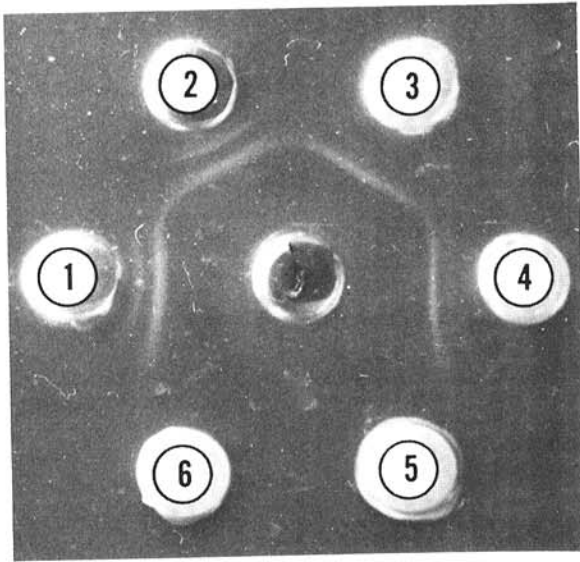


Fig. 5. Precipitation reaction by double-diffusion method between antiserum to CBB isolate 10.32L (center well) and CBB isolate 13.35L (1), 3.25L (2), 1.23L (3), and 11.33L (4), *Xanthomonas pruni* (5), and *X. manihotis* (6). Note that CBB isolates 13.35L and 3.25L formed two distinct rings of precipitate.

DL-phenylalanine was utilized by all isolates except 6.28L.

- 5) *Amines*. — Methylamine was utilized by all isolates except 2.24L and 3.25L. All isolates utilized acetamine, creatine, and N-acetyl-D-glucosamine, but no isolate could utilize L-histidine, histamine, benzylamine, or ethanolamine.

- 6) *Inorganic sources of N*. — CBB utilized potassium, sodium, and calcium nitrate, as well as ammonium sulfate. However, potassium nitrite was not utilized.

- 7) *Antibiotic sensitivity*. — All isolates of CBB were sensitive to dihydro-streptomycin (10  $\mu$ g), tetracycline (30  $\mu$ g), novobiocin (5  $\mu$ g), chloromycetin (5  $\mu$ g), rifampicin (30  $\mu$ g), bristaciin (30  $\mu$ g), kanamycin (5  $\mu$ g), neomycin (5  $\mu$ g), rifamicin (30  $\mu$ g), and sulfamethoxazole-trimethoprim (25  $\mu$ g). Only isolates 1.23L, 2.24L, and 14.36L were sensitive to penicillin (10 units); all others were resistant. Isolates 2.24L and 14.36L were sensitive to macrodantin (100  $\mu$ g), but all others were resistant. Isolate 13.35L was resistant to pentrexyl (25  $\mu$ g), but all others were sensitive to this antibiotic. Isolate 1.23L was resistant to erythromycin (15  $\mu$ g); all others were sensitive to this antibiotic. Only isolate 2.24L was resistant to gantrisin (1  $\mu$ g).

*Serological relationships*. — Antiserum for CBB isolates agglutinated with a flocculent precipitate when added to a suspension of  $1.5 \times 10^8$  cells/ml of any of the 14 isolates of CBB. Agglutination decreased to granular or microscopic granular as dilution of the antiserum increased. Two serological groups were evident among isolates of CBB (Table 1) when the double-diffusion method was

used. The first group, including isolates 3.25L, 7.29L, 9.31L, and 13.35L, was characterized by the formation of two distinct rings of precipitate around each well. The second group, which included all remaining isolates, was characterized by the formation of only one distinct ring of precipitate (Fig. 5).

Isolates of three species of *Erwinia*, two of *Pseudomonas*, and 10 of *Xanthomonas* did not agglutinate with CBB antiserum. Of six nonpigmented xanthomonads tested [*X. manihotis*, *X. zingiberi*, *X. proteomaculans*, *X. cannae*, *X. panici*, and *P. (Xanthomonas) rubrisubalbicans*, obtained from M. Starr, Dept. of Bacteriology, University of California, Davis] only *X. manihotis* induced a slight reaction which was noticeable only after 15 days of incubation. However, when a high concentration of bacterial cells ( $10^{10}$  cells/ml or higher) of *X. manihotis* was mixed 1:2 with CBB antiserum, no reduction in the strength of the precipitation reaction between the treated antiserum and other isolates of CBB was observed.

Additional serological tests with antiserum prepared with CBB isolates 1.23L and 9.31L gave similar results to those obtained with antiserum to isolate 10.32L.

*Typing with phage and Bdellovibrio sp.* — Plaques were obtained when the bacteriophage or *Bdellovibrio* sp. isolated from soil was used to inoculate isolates of CBB. No plaques were obtained on cultures of any of the three species of *Erwinia*, two of *Pseudomonas*, or 10 of *Xanthomonas* tested.

In comparative tests with the bacteriophage, three of the 14 isolates of CBB yielded a low number of plaques (5-10) per plate; each of the remaining isolates yielded more than 200 plaques per plate when inoculated with a standard phage suspension (see Methods).

Lysis induced by *Bdellovibrio* sp. was characterized by the presence of clear, round plaques noticeable 24 hr after inoculation of the bacterial host. As incubation increased, the lysed areas enlarged; borders of these areas were not defined and a clear halo was evident surrounding each plaque. Plaques induced by *Bdellovibrio* sp. were five times larger than those induced by the phage on the same bacterial culture.

**DISCUSSION.** — The syndrome induced by CBB on cassava is so complex that it appears to be unique among the known bacterial diseases of plants. CBB, along with several pseudomonad pathogens of deciduous fruit trees (13), can induce leaf spotting, shoot wilting, die-back, exudation of gum, and vascular discoloration. In susceptible cultivars, however, CBB also moves systemically through the stem and into the vascular tissues of the root, inducing discoloration and necrosis, in common with many vascular pathogens (4, 32). CBB appears to be restricted to the vascular strands, particularly in mature and old stems. Infection induced by CBB is more severe in, and is frequently limited to, the young tissues of the cassava plant, perhaps because mature vessel members have a thick, lignified



secondary wall, and the middle lamella may also become lignified, establishing a barrier that the enzymatic apparatus of CBB cannot overcome.

Once CBB has multiplied within the substomatal cavity, it invades and destroys the spongy mesophyll tissues. This explains the fact that lesions first become evident on the underside of the leaves. How CBB enters the vascular system is not known, but it moves readily from leaf lesions to the stem through the vascular tissues. It has been suggested (Pereira, *personal communication*) that some strains of *X. manihotis* induce symptoms on leaves only, and that others are associated with both stem lesions and shoot wilting. Our results suggest no such strain specificity. In fact, the uniform level of virulence among CBB isolates was indicative of very limited variability.

The two distinct serological reactions induced by isolates of CBB may be related to geographical origin. One of the two CBB isolates from Brazil showed two, rather than one, distinct rings of precipitate in the double-diffusion tests, a characteristic shared only by Colombian isolates from Monteria (9.31L), Dorada (3.25L), and Villavicencio (7.29L). Information obtained from the Instituto Colombiano Agropecuario (Perez, *personal communication*) indicates that, in 1965, vegetative material of cassava from Brazil (São Paulo) was introduced into Colombia (Monteria), and then distributed to growers. It may be significant, therefore, that isolates from this area in Colombia and that from São Paulo presented similar serological reactions.

Of the four known bacterial pathogens on cassava, *P. solanacearum*, *E. cassavae*, and *X. cassavae* are obviously not related to CBB. Disease symptoms reportedly induced by the first two pathogens, and the morphological, physiological, and biochemical characteristics (2, 5, 10, 19, 24) of each of these bacteria are very different from those of CBB. For instance, *X. cassavae* induces leaf spots, but these are initially circular, not angular, and are surrounded by a yellow halo with radial necrosis of the veins. In addition, the pathogen, unlike CBB, is restricted to leaf tissues and produces a yellow pigment on sugar-containing media (39).

The symptoms reportedly induced by *X. manihotis* on cassava (3, 14, 18, 34) are similar to those induced by CBB. However, several of the morphological, cultural, and biochemical characteristics of *X. manihotis* [as originally described by Burkholder (10)] are different from those of CBB. CBB cells are considerably smaller than those of *X. manihotis*. CBB cells have a singular polar flagellum, whereas *X. manihotis* is nonmotile (10). These two organisms also differ in H<sub>2</sub>S production, nitrate utilization, starch hydrolysis, and utilization of rhamnose and acetic acid as carbon sources.

When the culture of *X. manihotis* (ICPB-XM4) isolated by Burkholder in 1939 (10) was compared with CBB isolates, the following differences were found: ICPB-XM4 was only weakly pathogenic to cassava and was restricted to young stem tissues; cells were nonmotile and larger than those of CBB. The

generation time at 32 C for ICPB-XM4 was 92 min, whereas that of CBB was 46 min at the same temperature; the two species were not related on the basis of bacteriophage, *Bdellovibrio*, and serological typing. Serologically related substances were not removed by cross-absorption of CBB antiserum with *X. manihotis* cells. Moreover, CBB was not serologically related to *X. pruni*, although *X. manihotis* is reportedly related to this species (17).

On this basis, it could be concluded that CBB is different from *X. manihotis* as originally described by Burkholder. However, Pereira and Zagatto (34), working with a bacterium they called *X. manihotis*, reported that their isolates were motile, hydrolyzed starch, and did not produce H<sub>2</sub>S. These characteristics are common to all our isolates of CBB; in addition, their isolates caused disease symptoms identical to those caused by CBB. CBB and the bacteria isolated by the Brazilian workers are probably identical, but are sufficiently different from *X. manihotis* to be considered a separate strain, or perhaps a separate species.

The differences recorded between our cultures and the isolate of *X. manihotis* originally isolated by Burkholder may reflect changes that have occurred in the latter as a consequence of repeated subculturing over a period of more than 30 yr. Burkholder's isolate may have been a nonmotile variant when isolated. The differences in serological properties, however, are more difficult to explain. Further comparative studies of CBB and different isolates of *X. manihotis* from Brazilian sources are planned.

Because of the lack of yellow pigment formation by CBB, one may question the classification of this bacterium in the genus *Xanthomonas*. CBB possesses some of the characteristics of xanthomonads (8, 37), but, in addition to lack of pigmentation on sugar-containing media, it is a fast-growing bacterium, does not produce H<sub>2</sub>S, accumulates poly-β-hydroxybutyrate (PHB), and its optimum temp is higher than that of most xanthomonads. Nutritionally, it is more versatile than most xanthomonads. A few nonpigmented species, other than *X. manihotis*, have been included in the genus *Xanthomonas* (5), but CBB is not serologically related to these bacteria (*X. zingiberi*, *X. proteomaculans*, *X. cannae*, and *X. panici*). Cultural, physiological, and biochemical characteristics of the xanthomonads appear to be sufficiently diverse among isolates from different hosts (11, 15, 16, 38) to preclude any obvious basis to exclude CBB from the genus, however.

When CBB is compared with pseudomonad plant pathogens, it is possible to fit this pathogen in group III of Sands et al. (35). As with species in this group, CBB is nonpigmented, accumulates PHB, and grows at 37 C. PHB is characteristically accumulated by pseudomonads, but not by xanthomonads, except for *X. albilineans* (20). It appears that CBB and *P. solanacearum* (9, 12, 21, 37) are not closely related to the species currently included in the genus *Pseudomonas*, and may belong to a distinct taxonomic group of nonpigmented species which



produce slimy, mucoid, white colonies on sugar-containing media. Further research is needed to determine whether such a group is a taxonomic entity within the genus *Pseudomonas*. Future work on DNA-DNA homology and G/C ratios may help to resolve this problem. Until such information becomes available, it is suggested that CBB be referred to as a strain of *X. manihotis*. It is clear, however, that the taxonomic position of this bacterium will have to be revised in the near future.

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