Coffee Leaf Scorch Bacterium: Axenic Culture, Pathogenicity, and Comparison with *Xylella fastidiosa* of Citrus

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ABSTRACT

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Symptoms of coffee leaf scorch (CLS) appear on young flushes of field plants as large marginal and apical scorched areas on recently mature leaves. Affected leaves drop, shoot growth is stunted, and apical leaves are small and chlorotic. Symptoms may progress to shoot dieback. Only scorched leaves which could not be related to other known agents consistently contained bacteria and bacterial agglomerates when observed with light microscopy. Only plants with these symptoms were positive in enzyme-linked immunosorbent assay (ELISA) tests using antiserum to *Xylella fastidiosa* Wells et al. The bacterium *Xylella fastidiosa* Wells et al. was isolated in November 1995 from coffee (*Coffea arabica*) leaves with scorch symptoms on supplemented periwinkle wilt medium. Colonies were circular, dome-shaped, white, and 0.5 to 1.5 mm in diameter. Two of 10 young coffee seedlings stem-inoculated with a suspension of the isolated *X. fastidiosa* in January 1996 showed leaf scorch symptoms 3 to 5 months later, contained bacteria in xylem extracts, and reacted positively in ELISA using antiserum to the citrus variegated chlorosis (CVC) strain of *X. fastidiosa*. ELISA-positive bacteria were reisolated from this plant. None of the symptomless plants, including controls, revealed bacteria on microscopic examinations, ELISA, or isolation attempts. Antisera developed against cultured bacteria from both CLS and CVC plants reacted positively against plant extracts of both diseases in dot immunobinding assays (DIBA). The level of detection was about 5×10^5 bacteria ml^{-1} for both homologous and heterologous reactions. The polymerase chain reaction amplification products produced by CLS and CVC strains of *X. fastidiosa* were indistinguishable. Geographical distribution of these strains is not the same. CLS is widespread and usually occurs if coffee is adjacent to CVC-affected citrus. However, CVC does not always occur when citrus is grown adjacent to CLS-affected coffee. The bacteria are closely related, if not identical.

Additional keywords: amarelinho, *Citrus sinensis*, diagnosis, orange, PCR, requeima do café

Leaf scorching on coffee (*Coffea arabica* L.) plants was found in São Paulo, Brazil in 1995. Of the two principal coffee varieties grown in São Paulo, "Mundo Novo" and "Catuai Amarelo," the most severe symptoms were found in the latter. Symptoms begin with apical and marginal leaf scorch (Fig. 1), reduction in internode length of new flush, small, pale green to yellow leaves, shoot dieback, and overall plant stunting. Fruit size and yield are generally reduced. Symptoms are more evident during the winter, especially during periods of water stress. In the spring, with rain, affected leaves drop and plant appearance improves as new shoots are produced.

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Actual plant death due to the disease may take several years to occur.

Serological and polymerase chain reaction (PCR) tests indicated the presence of the bacterium *Xylella fastidiosa* Wells et al. in symptomatic coffee plants (20). Xylem extracts from affected leaves contained bacteria morphologically similar to *X. fastidiosa*, which reacted positively to antiserum prepared against an isolate of *X. fastidiosa* from citrus affected with citrus variegated chlorosis (CVC) (16). The bacteria were isolated in supplemented periwinkle wilt (SPW) culture medium (10), and the disease was named "requeima do café" or coffee leaf scorch (CLS). Similar isolations were later confirmed elsewhere (2). The economic significance of CLS is unknown. Extensive coffee plantations have been eliminated in São Paulo. CLS was not reported prior to 1995, possibly because leaf scorching was often attributed to other problems, including the coffee leaf miner (*Perileucoptera coffeella* Guein. & Meneville), coffee rust (*Hemileia vastatrix* Berk & Br.), water stress, or destruction of the root system by nematodes. It is now suspected that CLS may have been the problem, since many remaining coffee plants have tested positive for *Xylella*.

The objectives in this study were to isolate the CLS bacterium in vitro, to produce polyclonal antiserum against it, and to confirm its pathogenicity by fulfilling Koch's postulates. In addition, the distribution of the bacteria in infected plants and the serological relationship between the coffee and citrus bacterial strains were studied. The close serological relationship between the coffee and citrus strains of *X. fastidiosa* was confirmed by PCR assay.

MATERIALS AND METHODS

***X. fastidiosa* diagnosis.** Bacterial detection was carried out using light microscopy (LM), scanning electron microscopy (SEM), dot immunobinding assays (DIBA), and enzyme-linked immunosorbent assays (ELISA). We obtained xylem extracts for LM by the syringe method (15,17). Briefly, leaf petioles were excised and fitted to a 2- to 4-cm long by 0.2-cm diameter plastic tube attached to a syringe. Sterilized, distilled water was then pushed through the xylem by applying manual pressure to the syringe to release bacteria from the vessels. The resulting drops of extract were dried on glass slides, stained with methylene blue, and observed at 400× with a light microscope for the presence of bacilliform cells or their agglomerates typical of *X. fastidiosa* (6,11,24).

Immunofluorescence assay was carried out on syringe extracts as prepared above, or by using membrane entrapment of extracts (3). Four to five petioles were chopped in approximately 2 ml of 0.02 M Tris (pH 8.2) buffer and the supernatant was passed through a Nucleopore 25-mm Swin-Lok filter assembly (Nucleopore Corporation, Pleasanton, CA 94566) containing a 5.0-µm polycarbonate membrane for trapping cellular debris and a 0.2 µm membrane for trapping bacteria. The latter was then removed and incubated for 1 h in a 1:20 dilution of *X. fastidiosa*-specific immunoglobulin (IgG) prepared to the citrus variegated chlorosis (CVC) strain (ca. 1 mg ml^{-1}) in Tris-BSA-gelatin buffer (20 mM Tris, 0.9% NaCl, pH 8.2 containing 0.1% bovine serum albumin and 1% gelatin). After a wash in Tris-BSA-gelatin buffer, the membranes were incubated in tetramethyl-rhodamine isothiocyanate (TRITC)-labeled goat anti-rabbit IgG (1:20)

for 1 h. After washing, the membranes were mounted and viewed with an epifluorescence microscope using a 580 to 590 nm wavelength filter as previously described (3).

SEM observations were carried out by fixing root and stem xylem or whole petioles in Karnovsky solution for 48 h, post-fixing in 1% osmium tetroxide for 4 h, dehydrating in an acetone series, transferring to 100% ethanol, and drying on a Ladd critical point dryer using CO_2 as carrier fluid. The samples were then sectioned with a razor blade through the xylem, coated with gold/palladium using a Ladd sputter-coater, and viewed in a Hitachi S530 scanning electron microscope.

Extracts for both the previously described DIBA (13) and double-antibody sandwich (DAS)-ELISA (9,19,32) tests were prepared by cutting approximately 40 mg of excised tissue (root or stem xylem, or whole petioles) into pieces approximately 1 mm long. These were put into 400 µl of distilled, sterilized water and centrifuged at 500 × *g* for 2 min to help release bacteria into the supernatant.

Field symptoms and bacterial distribution. In order to relate leaf symptoms to the presence of *X. fastidiosa* in coffee, leaves showing symptoms both from unknown or known (coffee rust and coffee leaf miner) causes, as well as normal-appearing coffee leaves, both from symptomless branches in the field or healthy controls under screen, were analyzed by LM for the presence of bacteria. Sixteen to 30 leaves of each type were examined from two coffee blocks in Casa Branca, São Paulo. *X. fastidiosa* identification was confirmed on several samples by immunofluorescence. Bacteria were observed in both orange and coffee roots (2- to 5-mm diameter), stems (3- to 8-mm diameter), and leaf petioles examined by SEM. The bacteria were identified as *X. fastidiosa* by LM and ELISA tests. Greenhouse-grown 4- to 12-month-old coffee plants (from seed) were used as healthy controls. Scorched coffee leaves and orange leaves with CLS and CVC symptoms and bacteria in the petioles (LM) were used as positive controls.

Isolation, culture, and antibody production. Xylem extracts were obtained by surface-disinfesting petioles or veins from symptomatic leaves of coffee with CLS for 1 min in 70% ethanol and 3 min in 10% bleach, washing for 5 min in sterile distilled water, cutting approximately 1-mm pieces into 1 to 2 ml of SPW (10) broth, and centrifuging at 700 × *g* for 2 to 3 min to help release bacteria from the xylem. Supernatant aliquots of 1 ml were then inoculated in liquid or on solid SPW media and incubated microaerobically in the dark at 27°C. After bacterial colonies had grown and were confirmed to be *X. fastidiosa* by colony morphology and PCR, washed cells from pure cultures in phosphate buffered saline with 0.001% sodium azide were sent to Cocalico Biologicals, Inc. (Reamstown, PA) for antisera production in rabbits. The IgG fraction of the serum was isolated by protein A column chromatography (18), adjusted to approximately 1 mg ml^{-1}, and an aliquot conjugated with alkaline phosphatase, using glutaraldehyde. Reactivity was then tested using immunofluorescence as described above, DIBA at a doubling dilution series from 1:10,000 to 1:160,000, and ELISA at 1:2,000. Test bacteria were from culture and were tested in a 10-fold dilution series from ~10 to ~10^8 bacteria ml^{-1}.

Pathogenicity. Bacteria were isolated on SPW medium from scorched coffee leaves collected at Casa Branca, São Paulo, as described above, and subcultured once in the same medium. A log-phase cell suspension was obtained by growing 10 to 14 days in SPW media and was collected by centrifugation at 3,500 × *g* for 5 min, followed by resuspension of the pellet with sterilized distilled water to a concentration of ~10^9 cells per ml. A flap of stem tissue was raised on the stem of 2-month-old coffee plants, cv. Mundo Novo, by cutting tangentially upward with a razor blade, exposing the wood. Drops of bacterial suspension were then continuously applied underneath the flap with a pipette until 100 µl or ~10^8 cells per plant had been taken up in approximately 2 h. Ten plants were inoculated on 11 January 1996, and the same number of control plants were mock-inoculated with water or not inoculated. A few drops of methylene blue were added to the water in one control plant to indicate translocation, showing uptake to all plant extremities above the inoculation point. Detection of bacteria by LM and ELISA and reisolation attempts were carried out 80 and 105 days after inoculation.

Bacterial size, serology, and PCR assay. One hundred bacterial cells from three coffee petiole samples were measured using a light microscope with a reticulated ocular calibrated to a template slide.

Purified rabbit IgG prepared against cultured *X. fastidiosa* from CLS was compared with purified rabbit IgG prepared against cultured *X. fastidiosa* from CVC in homologous and heterologous immunofluorescence and DIBA tests. The dilution end-point was determined for the dot assay by counting bacteria in 1 µl-aliquots and preparing fivefold dilution series starting at ~5×10^7 bacteria ml^{-1}.

X. fastidiosa strains 96-1, isolated from CLS-diseased coffee, and 10T, isolated from CVC-diseased citrus, were grown to stationary phase in SPW broth. Cells were washed twice and resuspended in water. Cells were added to PCR master mix in a 10-fold dilution series starting at ~5×10^6 cells. Amplification conditions were as previously described (22) using the primer pair 272-2 and CVC-1. Electrophoresis of amplification product was at 100V through 2% NuSieve (FMC, Rockland, ME) agarose gels.

RESULTS AND DISCUSSION

***X. fastidiosa* diagnosis.** *X. fastidiosa* was easily observed by SEM in CLS-affected coffee and CVC-affected citrus rootlets and small branches. This bacterial presence was confirmed by LM and DAS-ELISA. Similar bacterial distribution was found in citrus in a previous study (15). *X. fastidiosa* also has been observed in both branches and roots in other plants, including peach (1,31). Therefore, *X. fastidiosa* can be distributed throughout an entire plant, and must be moved downward to the root system, since the sharpshooter leafhopper vectors are known to feed exclusively on aerial plant parts (23). When viewed by SEM, bacteria were observed colonizing the lumen of the xylem vessels (Fig. 2), frequently resulting in plugs composed of bacteria imbedded in a matrix apparently of plant origin, as described for Pierce's disease of grapes (8,11,12,24). Based on SEM observations, the bacteria in roots, stems, and petioles of coffee and citrus were indistinguishable, and had characteristics similar to those previously described in citrus (5,11,26) as well as in other plants (4,14,24,28,29).

Field symptoms and bacterial distribution. Twenty-nine of 30 coffee leaves with scorch symptoms had many bacteria in xylem extracts, observed with the LM as scattered cells or typical bacterial agglomerates. None of the leaves from control plants grown from seed under screen con-

Fig. 1. Symptoms of coffee leaf scorch on coffee (*Coffea arabica* L. cv. "Mundo Novo").

tained bacteria. Presumptive *X. fastidiosa*, however, is widespread in plants exposed in the field, since 10% of symptomless leaves also had bacteria. These bacterial populations in asymptomatic plants may indicate recent infections. Alternatively, they may represent field infection by strains of *X. fastidiosa* not related to CLS. Some leaves with scorch-like symptoms that could be partially attributed to coffee leaf miner or coffee rust also had bacteria (25 and 14% of leaves sampled, respectively). Symptoms of CLS could be masked in those leaves by the other disease or pest symptoms. Plants with scorched leaves showing bacteria in the xylem also had a wide range of decline symptoms. Some had nearly healthy appearance with just a few symptomatic leaves. More commonly, as the number of scorched leaves increased, defoliation and reduction in plant and leaf size were noticeable. In extreme cases, most branches had retained only the most distal leaves. These were typically small, chlorotic, and scorched, and the plants were severely stunted. These plants contained many bacteria and bacterial agglomerates in xylem extracts from roots, stems, and leaves, easily observed by LM.

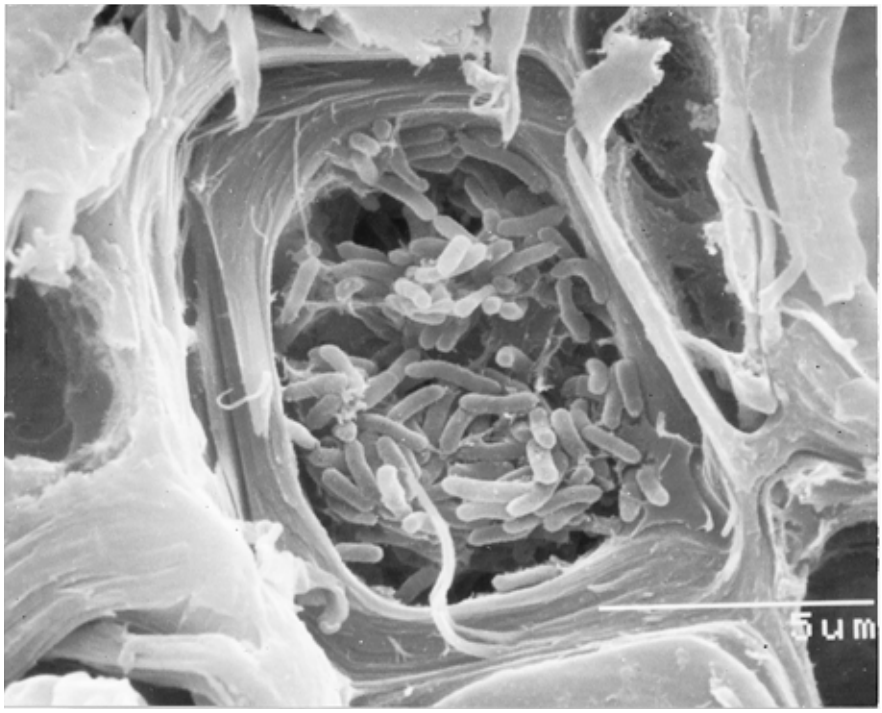

Fig. 2. *Xylella fastidiosa* bacteria in a cross-section of petiole xylem vessel affected by coffee leaf scorch as seen by scanning electron microscopy.

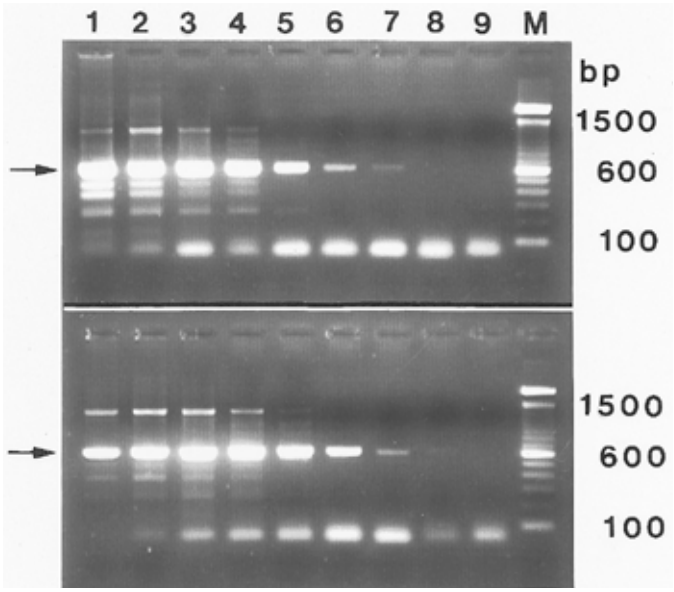

Fig. 3. Polymerase chain reaction (PCR)-amplification products produced by *Xylella fastidiosa* isolated from coffee leaf scorch-diseased coffee (top) and from citrus variegated chlorosis-diseased citrus (bottom). Bacteria were added to PCR in 10-fold dilution series beginning with ~5×10^6 cells per reaction in lane 1. Lane 9 contained the deionized water-only negative control, and lane "M" contained a 100-bp ladder as a size standard.

Isolation, culture, and antibody production. Isolation was successfully completed from 6 of 6 petioles of coffee leaves with CLS symptoms. Slow-growing bacterial colonies were observed approximately 20 days after inoculation. The colonies were circular, dome-shaped, white, and were 0.5 to 1.5 mm in diameter after 30 days. Morphological characteristics of the bacteria were similar to those of CVC *X. fastidiosa* (17), and other *X. fastidiosa* (29). Isolated bacteria repeatedly reacted positively in ELISA tests using antiserum to *X. fastidiosa*. Purified IgG from the polyclonal antiserum reacted positively to xylem extracts of CLS-affected plants in immunofluorescence tests at 1:20 dilution. The same extracts, as well as *X. fastidiosa* isolated from them, reacted positively in ELISA at 1:2,000, and in DIBA at all dilutions tested, from 1:10,000 to 1:160,000. Dilution endpoint was determined for the ELISA at ~10^5 bacteria ml^{-1}.

Pathogenicity. Two of 10 coffee plants inoculated with a suspension of *X. fastidiosa* from CLS-infected coffee leaves developed scorched leaf symptoms after 3 months. Bacteria with the characteristic morphology of *X. fastidiosa* were observed by LM in xylem extracts from a scorched leaf from these plants. The average ELISA reading for a mid-vein extract of the plants, using an antiserum against CVC *X. fastidiosa*, was 0.347, compared with 0.060, 0.050, and 0.930 for a healthy coffee plant extract, a water blank, and a positive CLS-infected coffee plant extract, respectively. *X. fastidiosa* was observed in culture 15 to 20 days after inoculating leaf vein extracts into SPW broth. LM observations of the isolated bacteria revealed morphological characteristics similar to the inoculated *X. fastidiosa*. The average ELISA results for both cultures were also positive (1.519), compared to 0.062 for a healthy coffee plant extract, 0.070 for a water blank, and 1.002 for a positive CLS plant. The successfully inoculated plants continued to have CLS symptoms and tested positive again for the pathogen in September 1996 using PCR assay (22). None of the control plants or the remaining inoculated plants without scorch symptoms had bacteria detectable with LM examination of xylem extracts, or positive ELISA readings or PCR assays. We therefore fulfilled Koch's postulates relating *X. fastidiosa* and "requeima do café" or coffee leaf scorch. The low level of successful inoculation of cultured bacteria into plants has been found for some tree strains of *X. fastidiosa* (10,30). Recently field observations indicate that cv. Mundo Novo may be more tolerant to CLS symptoms than cv. Catuai Amarelo. This difference may further ac-

count for the low level of inoculation success we obtained in pathogenicity tests.

Bacterial size, serology, and PCR assay. The rod-shaped bacteria extracted from CLS-affected petioles had dimensions of 0.4 µm ± 0.02 µm by 2.5 µm ± 0.5 µm dimensions, similar to *X. fastidiosa* from citrus (17) as well as from other plants (11,24).

Purified rabbit IgG against *X. fastidiosa* cultured from both CVC- and CLS-affected plants reacted indistinguishably to both homologous and heterologous extracts in immunofluorescence and DIBA tests. The DIBA dilution endpoints were the same for all reaction and cross-reaction combinations, i. e. $\sim 5 \times 10^5$ bacteria ml^{-1}.

The PCR amplification products produced by the CVC and CLS strains of *X. fastidiosa* were indistinguishable, and both strains had the same dilution endpoint in the PCR assay (Fig. 3). This is significant because this primer pair did not directly amplify any product from any *X. fastidiosa* strain from any host except citrus (22). In the previous work, all of the strains tested, except for the citrus strains, were of North American origin. The *X. fastidiosa* strains tested previously from Brazil, all from CVC-diseased trees, also were readily distinguished by random amplified polymorphic DNA (RAPD)-PCR analyses from the North American strains of *X. fastidiosa* (21). Thus, the coffee and citrus strains of *X. fastidiosa* are distinct from the North American strains.

All available data is consistent with hypothesis that the coffee and citrus strains of *X. fastidiosa* in São Paulo are closely related or identical to each other. Since the same leafhopper vectors of CVC commonly are present in coffee plantations in São Paulo, it is possible that strains from a natural endemic population in other hosts were challenge-inoculated into each crop until virulent strains were selected. It is common for *X. fastidiosa* strains to be inoculated and grow in different plants, behaving as pathogens in one or more of the host species (7,11,24). Geographical distribution of the coffee and citrus strains, however, does not appear to be congruent, since CLS is easily detected adjacent to healthy-appearing orange groves and in areas where CVC has not been reported or occurs at low levels (16,27). In addition, since coffee is propagated via seeds, and *Xylella*-related diseases are not seed-transmitted (11,24), the dissemination in coffee throughout the state of São Paulo must have occurred through the leafhopper vectors and, therefore, does not seem to be recent. In contrast, CVC has been rapidly disseminated by contaminated budwood. The detection of CVC in orange trees was officially reported in 1987 and probably was observed a few years earlier (25). This evidence suggests that different strains of *X. fastidiosa* may be involved in the coffee and the orange diseases, or the strains may be identical. We have initiated such cross-inoculation experiments to further evaluate the relation between these strains.

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