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Culture and Serological Detection of the Xylem-Limited Bacterium Causing Citrus Variegated Chlorosis and Its Identification as a Strain of *Xylella fastidiosa*

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Abstract. A xylem-limited bacterium resembling *Xylella fastidiosa* has been shown previously by electron microscopy to be associated with citrus variegated chlorosis (CVC), a new disease of sweet orange trees in Brazil. A bacterium was consistently cultured from plant tissues from CVC twigs of sweet orange trees but not from tissues of healthy trees on several cell-free media known to support the growth of *Xylella fastidiosa*. Bacterial colonies typical of *X. fastidiosa* became visible on PW, CS20, and PD2 agar media after 5 and 7–10 days of incubation, respectively. The cells of the CVC bacterium were rod-shaped, 1.4–3 μm in length, and 0.2–0.4 μm in diameter, with rippled walls. An antiserum against an isolate (8.1.b) of the bacterium gave strong positive reactions to double-antibody-sandwich (DAS), enzyme-linked immunosorbent assay (ELISA) with other cultured isolates from CVC citrus, as well as with several type strains of *X. fastidiosa*. This result indicates that the CVC bacterium is a strain of *X. fastidiosa*. ELISA was also highly positive with all leaves tested from CVC-affected shoots. Leaves from symptomless trees reacted negatively. Sweet orange seedlings inoculated with a pure culture of the CVC bacterium supported multiplication of the bacterium, which became systemic within 6 months after inoculation and could be reisolated from the inoculated seedlings. Symptoms characteristic of CVC developed 9 months post inoculation.

Citrus variegated chlorosis (CVC) was first observed in 1987 on sweet orange trees in the southwestern part of Minas Gerais, Brazil. Since then, the disease has been observed in the neighboring State of Sao Paulo. Rossetti et al. were the first to show by electron microscopy that a xylem-limited bacterium, probably a strain of *Xylella fastidiosa*, was present in all symptomatic leaves and fruits tested but not in similar tissues from symptomless trees [16]. Chagas et al. [3] confirmed these results. While both reports strongly suggest that *X. fastidiosa* may be the causal agent of CVC, fulfilment of Koch's postulates requires that the CVC bacterium be obtained in pure culture. We report here the cultivation of the CVC bacterium in cell-free media, the production of an antiserum against the cultivated bacterium, the close serological relatedness of the CVC bacterium with *X. fastidiosa*, and the establishment of an ELISA system for the detection of the CVC bacterium in citrus tissues. Congress abstracts on CVC have appeared [2, 11, 14].

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Materials and Methods

Plant materials. Twigs (about 3–5 mm in diameter) with attached leaves from symptomatic and asymptomatic sweet orange (*Citrus sinensis* (L.) Osb.) trees were collected and shipped to the Villenave d'Ornon (Bordeaux) laboratory. Four samples of "Natal" sweet orange on "Cleopatra" mandarin (three from symptomatic trees and one from an asymptomatic tree), collected on April 14, 1992, in Colina, S.P., Brazil, were received in the laboratory on April 21, 1992. Six other samples of "Valencia" sweet orange on rangpur lime, collected only from symptomatic trees on May 21, 1992 in Macaubal, S.P., Brazil, were received on May 25, 1992. Samples were kept at 5°C before culture of the bacterium was attempted. Finally, for blind testing, 20 midrib samples for ELISA were received from Brazil and Argentina in October 1992 and kept at −20°C before use.

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Culture media. Five media were prepared for the primary cultivation of the CVC bacterium from twigs of sweet orange trees. Media CS20 and PD2 were prepared according to [4] and [7], respectively. Medium PW [8] was slightly modified in that $MgSO_4 \cdot 7H_2O$ was added with other inorganic salts before autoclaving, and the stock solution of bovine serum albumin was prepared at 10%. CVC1 medium was prepared by dissolving in 970 ml of deionized water the following ingredients before autoclaving for 15 min: bacto-peptone (Difco), 4.0 g; tryptone (Difco), 1.0 g; K_2HPO_4, 1.2 g; KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; phenol red (0.2%), 10.0 ml; and agar, 12.0 g. Before plates were poured, two Millipore-filtered (0.45-μm pore size) stock solutions were added: 50 ml of glutamine (8%) and 60 ml of bovine serum albumin fraction V (10%). CVC2 medium contained the same ingredients and was prepared in the same way as CVC1, except that 10.0 ml of hemin chloride (0.1%) was added before autoclaving. The pH of both CVC1 and CVC2 media was about 6.5 without adjustment.

Culture of the CVC bacterium. The four "Natal" sweet orange samples (designated #1, #3, and #4 collected from symptomatic trees, and #5 from the asymptomatic tree) received in Villenave d'Ornon on April 21, 1992 were used for the culture assay on April 23 with the CVC1 and CVC2 media. Six other samples from symptomatic "Valencia" sweet orange trees (designated #6, #7, #8, #9, #10, and #11), received on May 25, were used for the culture assay the same day, with PW, CS20, PD2, CVC1, and CVC2 media. Four twigs each from samples #1, #3, and #4, three twigs from #5 and two twigs each from #6 through #11 were selected for isolation. All twigs (about 5–6 cm in length each) were surface sterilized in 1.06% sodium hypochloride for 15–20 min rinsed three times in sterile deionized water, and air-dried in a laminar flow hood. Each twig was cut, as finely as possible, into 1 ml of CVC1 broth medium. The suspension was collected in a test tube, agitated briefly, and dropped loopfully onto duplicate plates (5 drops of about 5 μl each per plate) of each medium. The inoculated plates were kept in plastic bags to prevent desiccation and were incubated at 30°C. Plates were observed for colony development at weekly intervals for a month with a binocular microscope.

Production of Antiserum. One of the colonies obtained from sample 8, twig 1, on medium PW was called 8.1.b. A 500-ml broth culture of isolate 8.1.b of the CVC bacterium in medium PW was centrifuged at 20,000 *g* for 30 min. The resulting pellets were washed twice in PBS buffer, centrifuged as above, and the final pellet was resuspended in 2 ml of PBS. Three intramuscular injections were performed on a rabbit at 10-day intervals, each with 400 μl (300 μg of proteins) of CVC cells in the presence of an equal volume of either Freund's complete (1st injection) or incomplete (2nd and 3rd injections) adjuvant. Blood was recovered 1 week after the last injection from the ear vein. The serum was collected, centrifuged at 1500 *g*, and filtered through 0.2-μm Millipore filters. Immunoglobulins (Ig) were purified from the serum by ammonium sulfate precipitation and affinity chromatography on protein A sepharose according to Ball et al. [1]. One mg of conjugate for DAS-ELISA was obtained by labeling with alkaline phosphatase (AP) [13].

Double-antibody-sandwich, enzyme-linked immunosorbent assay (DAS-ELISA). DAS-ELISA was done according to Clark and Adams [6]. Four different concentrations of the coating Ig, namely, 1, 5, 10, and 20 μg/ml, were tested with three different dilutions of AP conjugate, namely, 1/500, 1/1000, and 1/2000. PBS was used as control for each Ig AP conjugate combination. Plates were coated with Ig at the mentioned concentrations and incubated at 37°C for 4 h. After three washes with PBS-tween buffer, the antigen was added at a protein concentration of 30 μg/ml, and the ELISA plate was incubated overnight at 4°C. The AP conjugate at the indicated dilutions was added after three washes with PBS-tween buffer, and the plate was incubated at 37°C for 4 h before substrate (paranitrophenyl phosphate) at 1 mg/ml was added. The readings of the optical density at 405 nm were recorded 15 and 30 min after the addition of substrate.

On the basis of the above experiments (see Results), DAS-ELISA for detection of the CVC bacterium was carried out with a concentration of 5 μg/ml of Ig for coating the ELISA plates (4 h at 37°C) and with a 2000-fold dilution of the AP conjugate (4 h at 37°C). All antigens were prepared in PBS buffer. Plant extracts were obtained by grinding 1 g of leaf midribs in 3 ml of PBS buffer in a Polytron homogenizer. The extract was filtered through four layers of cheesecloth, and 150 μl of filtrate was placed in each well of the ELISA plate overnight at 4°C.

Eight type strains of *X. fastidiosa* obtained from the American Type Culture Collections (ATCC) and five strains originally isolated from grapevines in Georgia with Pierce's disease symptoms were compared with the CVC bacterium by ELISA. Cell suspensions of each strain used as antigen were prepared by suspending scraped 10-day-old cultures from one plate into PBS buffer and adjusting the suspension to a Klett-Summerson colorimeter reading of 50. An aliquot of 150 μl of the antigen preparation was used per well of the ELISA plate.

For further comparison, all cell suspensions used above were reacted with an antiserum produced against *X. fastidiosa* manufactured by Agdia, Inc., (Elkhart, Indiana). The Agdia 1000 Reagent set (Catalog number EP345) used peroxidase-conjugated IgG for ELISA tests, and the optical density was recorded at 410 nm.

Cellular Morphology and Ultrastructure. Cells of a 10-day-old culture of isolate 8.1.b were used for electron microscopic observations. Negative staining of cell suspensions was done by suspending scraped cells from PW agar plates in 1 ml PBS. A drop of cell suspension was pipetted on a Formvar-coated copper specimen grid. After 5 min, the excess culture broth was removed with filter paper, and a drop of 2% aqueous ammonium molybdate was placed on the coated grid for 1 min, then removed with filter paper. The grids were examined in a Philips CM 10 transmission electron microscope.

Inoculation of the CVC bacterium into sweet orange seedlings. Twelve Hamlin sweet orange seedlings were mechanically inoculated, by stem injection, on June 4, 1992. Eight seedlings were injected with 50–250 μl of a 10-day-old broth culture of the CVC bacterium in PW medium; four seedlings received equivalent amounts of uninoculated PW medium. The multiplication of the bacterium in the seedlings was monitored by DAS-ELISA on August 26 and November 5, 1992. Reisolation of the CVC bacterium from two inoculated seedlings was attempt in January 1993 on PW medium as described above.

Results

Culture of the bacterium associated with CVC. In the first isolation attempt, four out of nine symptomatic twigs yielded bacterial colonies on medium CVC1,

and two out of nine on medium CVC2 after an incubation period of 14 days; no colonies were obtained from asymptomatic healthy twigs. In the second isolation attempt, the recovery of the bacterium was 100% on all five media used, even though various incubation periods were needed on different media. On media PW and PD2, all 12 symptomatic twigs yielded colonies after 9 days of incubation, while on media CS20, CVC1, and CVC2 25 days were required for colony development from certain twigs. In subsequent isolation experiments, similar high recovery rates of the bacterium from diseased tissues were observed. Except for obvious contaminants, no colonies have ever been obtained with plant material from healthy trees. The bacterium consistently isolated from tissues showing CVC symptoms will be designated as the CVC bacterium.

It took approximately 2 weeks before the colonies became visible under a binocular microscope on CVC1 and CVC2 media. The average number of colonies obtained from one loopful of tissue homogenate was 22.5 on medium CVC1 compared with 14.5 on medium CVC2.

When three additional media were used in subsequent isolation attempts, it was obvious that the CVC bacterium preferred PW, PD2, and CS20 to CVC1 and CVC2. The bacterial colonies became visible in 5 days on PW, in 7–10 days on PD2 and CS20, and in 14 days on CVC1 and CVC2.

Morphology of the CVC bacterium. The morphology of the CVC bacterium grown on PW agar is shown after negative staining (Fig. 1). The bacterium is rod-shaped, 0.2–0.4 μm in diameter, and 1.4–3 μm in length; it has a rippled wall and divides by binary fission.

Detection of the CVC bacterium by ELISA *Conditions for the DAS-ELISA*. The conditions for the ELISA were evaluated with different concentrations of Ig for coating the ELISA plate and different dilutions of the AP conjugate with CVC cells as antigen. The results of the various combinations are shown in Table 1. When coating was done with a concentration of 20 μg/ml of Ig, high optical densities (OD) were obtained with the CVC bacterium (>2 after 30 min), but the control wells with PBS buffer were also high (OD 0.2–0.6). At lower concentrations of Ig for coating, the OD of the control wells was generally below 0.1, and high OD readings were obtained with the CVC bacterium. From these results, we finally chose a concentration of 5 μg/ml for coating the ELISA plate and a 2000-fold dilution of the AP conjugate.

Fig. 1. Negative staining of cultured cells of the **CVC** bacterium; arrows indicate bacteria dividing by binary fission. Bar represents 1 μm.

Detection of cultured cells of the CVC bacterium. The titer of the antiserum produced against isolate 8.1.b of the CVC bacterium was high. The reading of the optical density at 405 nm was >2 at 30 min when a concentration of the homologous antigen of 1.2 μg protein/ml was used. The reading was still significant (0.238) when the antigen concentration was as low as 0.035 μg/ml (Table 2). The other isolates from CVC-infected material also reacted positively. No reaction was noticed with *Escherichia coli* cells at a concentration of 5 μg/ml.

Detection of the CVC bacterium in plant tissues. The antiserum raised against isolate 8.1.b detected the CVC bacterium in homogenates of leaf midribs prepared by homogenizing 1 g of midribs in 3 ml of buffer. Optical densities higher than 2 were often obtained when midribs of leaves showing CVC symptoms were used. Midribs from healthy sweet orange trees or greening-affected trees grown in the Bordeaux greenhouse, or from asymptomatic trees in CVC-affected orchards or orchards without CVC, gave OD readings close to zero. More recently, midribs purposely shipped without indications of disease status were received from Brazil for blind testing. All samples giving positive ELISA reactions turned out to be from symptomatic trees, while all negative reactions were given by trees from CVC-free orchards whether they were in CVC-affected or in CVC-free regions. Positive reactions were ob-

Table 1. Conditions for the DAS-ELISA

Dilution of AP conjugate	Antigen	Concentration of Ig (μg/ml) 1		5		10		20	
		15 min	30 min	15	30	15	30	15	30
		[a]	[b]	[a]	[b]	[a]	[b]	[a]	[b]
1/500	CVC[c]	0.415	(>2)	1.361	(>2)	0.399	(>2)	1.184	(>2)
	PBS	0.014	(0.05)	0.08	(0.3)	0.03	(0.05)	0.11	(0.6)
1/1000	CVC	0.261	(>2)	0.612	(>2)	0.293	(>2)	0.576	(>2)
	PBS	0.06	(0.06)	0.02	(0.2)	0.03	(0.05)	0.07	(0.5)
1/2000	CVC	0.167	(0.718)	0.328	(>2)	0.132	(0.912)	0.267	(>2)
	PBS	0.015	(0.04)	0.03	(0.06)	0.03	(0.05)	0.04	(0.2)

[a,b] The OD at 405 nm was read 15 min ([a]) and 30 min([b]) after addition of substrate.
[c] Cultured cells of the CVC bacterium.

Table 2. Sensitivity of DAS-ELISA for the detection of cultured cells of the CVC bacterium

Antigens	Concentration (μg protein/ml)	OD at 405 nm after 30 min
PBS buffer		0.006
CVC bacterium:		
8.1.b isolate	5	>2
	2.5	>2
	1.2	>2
	0.62	0.995
	0.31	1.601
	0.15	0.674
	0.075	0.359
	0.035	0.238
	0.017	0.084
8.1.a isolate	ND[a]	1.905
8.2.a isolate	ND	1.860
9.1.b isolate	ND	>2
Escherichia coli	5 μg protein/ml	0.083

[a] ND, not determined.
Proteins were determined according to Lowry et al. [12].

tained even with midribs of symptomless leaves but collected on trees with CVC-affected fruits.

The above ELISA reactions were obtained in Bordeaux with shipped plant material. ELISA reactions were also carried out by us, early September 1992, in the state of Sao Paulo with freshly collected material. All symptomatic sweet orange trees tested (17 trees representing 34 samples) gave strong positive ELISA reactions with symptomatic leaves or asymptomatic leaves from the same shoot, in all areas where the disease occurs (Barretos, Cocal, Taquaritinga, Catigua), and whatever the sweet orange varieties affected (Pera, Natal, Valencia, Hamlin) irrespective of rootstocks (*Citrus volkameriana*, Rangpur lime, "Cleopatra" mandarin). ELISA was negative with leaf midribs from symptomless trees in CVC-affected orchards or from tree in areas still free of CVC (Sete Lagoas orchard, Moji Guaçu). Roots with a diameter of 1–2 mm and 5–6 mm from symptomatic trees were also assayed; they gave negative reactions.

Finally, leaves with symptoms of Pecosita disease, very similar to those of Brazilian CVC, were received from Argentina (Tabay, Corrientes). They gave strongly positive ELISA reactions (OD above 2), indicating that the disease is present not only in Brazil but also in Argentina and that CVC and Pecosita diseases are the same.

Serological relatedness of the CVC bacterium and X. fastidiosa. The antiserum produced against the CVC bacterium gave high OD readings with several *X. fastidiosa* strains, and especially those from grapevine affected with Pierce's disease and almond affected by leaf scorch (Table 3, left column). These strains belong to group I of the *X. fastidiosa* strains, as described by Purcell [15]. In group II, the ragweed stunt strain gave a strong reaction, but the periwinkle, mulberry, and oak strains did not react (Table 3).

When the same strains were tested with the Agdia kit (Table 3, right column), the four isolates of the CVC bacterium reacted positively, but the ODs were much lower than with the CVC-specific serum. All Pierce's disease strains, as well as the ragweed stunt isolate, reacted positively, and the respective ODs were similar to those obtained with the CVC antiserum. The two strains of mulberry leaf scorch and that of periwinkle wilt, which did not react with the CVC antiserum, gave slightly positive reactions

Table 3. Serological relatedness between the CVC bacterium and strains of *Xylella fastidiosa*

Antigens tested	ATCC number	Antiserum produced against: CVC-8.1.b OD at 405 nm after 30 min	FXLB[a] OD at 410 nm after 30 min
CVC bacterium isolates:			
8.1.a	None	1.905	0.58
8.1.b	[b]	>2	0.75
8.2.a	None	1.860	0.87
9.1.b	None	>2	0.78
Xylella fastidiosa from:			
Mulberry leaf scorch	35868	0.057	0.20
Mulberry leaf scorch	35869	0.032	0.15
Oak leaf scorch	35874	0.045	0.96
Ragweed stunt	35876	>2	1.48
Periwinkle wilt	35878	0.07	0.11
Almond leaf scorch	35870	>2	1.48
Pierce disease of grape	35879	>2	1.48
Pierce disease of grape	35881	>2	1.48
Pierce disease of grape, Georgia isolates:			
chateau 3C	None	>2	1.48
112.V1	None	>2	1.48
116.V6	None	>2	1.48
116.V11	None	>2	1.48
MS7	None	>2	1.48
Escherichia coli		0.083	ND[c]

[a] FXLB = fastidious xylem-limited bacteria. ELISA kit (Agdia 1000 reagent set, catalog number EP345) provided by Agdia, Inc., (Elkhart, Indiana).
[b] Sent to ATCC but no number yet.
[c] ND = not done.

(OD 0.11–0.2) with the Agdia serum. The oak leaf scorch strain was the only one showing significant differences in reactions with the two antiserum. It was not detected with the CVC-specific antiserum but gave high OD (0.96) with the Agdia serum.

Detection and culture of the CVC bacterium after injection into sweet orange seedlings. Five out of eight seedlings inoculated with the CVC bacterium on June 4, 1992 gave positive DAS-ELISA reactions when tested on August 26 with the CVC antiserum (OD between 0.395 and >2), at a time when all seedlings were still symptomless. Later on, in October, chlorosis symptoms developed on a few leaves of certain seedlings. All seedlings were tested again on November 26. One additional seedling was positive in DAS-ELISA, and the bacterium had become systemic in all six positive seedlings, as it was present in newly developed terminal leaves located far from the inoculation points. Seedlings inoculated with PW medium alone gave negative DAS-ELISA results both in August and November and were symptomless. Isolation of the CVC bacterium on PW medium was done from two inoculated seedlings on which pronounced symptoms of variegated chlorosis had developed in January 1993. Colonies of the CVC bacterium could be observed on PW medium 5 days after inoculation.

Discussion

It was shown previously by electron microscopic observations [16] that a xylem-limited bacterium was present in CVC-affected leaves and fruits. This bacterium was morphologically similar to *X. fastidiosa*, a well known xylem-limited bacterium and the causal agent of several diseases of plants [5, 9, 10, 15, 17].

We have now been able to consistently culture a bacterium from CVC-affected tissues. That this CVC bacterium and the bacterium seen by electron microscopy are the same is supported by the following facts: (1) both occur in CVC-affected citrus, (2) the bacteria as seen in situ in the xylem and in the

cultures have the same size and morphology, and (3) the antiserum raised against the CVC bacterium gives highly positive DAS-ELISA reactions with CVC-affected leaves. Strong positive ELISA reactions were obtained with CVC-affected tissues from all the areas where CVC is present.

The following data indicate that the CVC bacterium is a strain of *X. fastidiosa*: (1) the two organisms are both xylem-limited, (2) they have the same morphology in planta and in vitro, (3) they grow in media developed for *X. fastidiosa*, (4) they have close serological relationships with other strains of *X. fastidiosa*, and (5) they are reported to have similar protein patterns upon polyacrylamide gel electrophoresis [14]. The serological reactions of the CVC-specific serum with other *X. fastidiosa* strains indicate that the CVC bacterium is more closely related to group I than to group II strains even though good reactions are observed with the Ragweed stunt strain. This is also evidenced by the ability of the CVC bacterium to grow in both PW and PD media.

In order to fulfill Koch's postulates, sweet orange seedlings were mechanically inoculated with the CVC bacterium. The CVC bacterium could be detected by DAS-ELISA in these plants 3 months after inoculation, had become systemic 4 months after inoculation, and could be reisolated from the inoculated seedlings. Symptoms of chlorotic variegation characteristic of the disease started to develop 6 months after inoculation and were conspicuous 3 additional months later. These results indicate that the CVC strain of *X. fastidiosa* is the causal agent of CVC.

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