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Authors

Chang, C. J.
Garnier, M.
Zreik, L.
et al.

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Citrus Variegated Chlorosis: Cultivation of the Causal Bacterium and Experimental Reproduction of the Disease

C. J. Chang, M. Garnier, L. Zreik, V. Rossetti and J. M. Bové

ABSTRACT. A xylem-limited bacterium associated with citrus variegated chlorosis (CVC) in Brazil was cultured on a modified PW medium on which the bacterium grew within 5 days. Electron microscopy of the cultured bacterium revealed a morphology similar to that observed *in situ* in the xylem of infected plants and resembling that of *Xylella fastidiosa*. The bacterium was 0.2-0.4 μm in diameter and 1.4-3 μm in length. Mechanical inoculation of the cultured bacterium into sweet orange seedlings resulted in a high concentration of the bacterium in the xylem of inoculated plants 3 months after inoculation. The bacterium 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 months later. These results indicate that this bacterium is the causal agent of the disease.

Citrus varigated chlorosis (CVC) is a new disease of citrus which appeared in the southern part of Minas Gerais, Brazil in 1987. It is now also present in the state of Sao Paulo. In 1990, we showed by electron microscopy that a xylem-limited bacterium resembling *Xylella fastidiosa* was associated with the disease (6). In order to fulfill Koch's postulates, we have tried to culture the bacterium in cell free media and to reproduce the disease by inoculation of the cultured bacterium into sweet orange seedlings.

MATERIAL AND METHODS

Plant material. Twigs (about 3-5 mm in diameter) with attached leaves from symptomatic and asymptomatic sweet orange trees were collected and shipped to the laboratory in Bordeaux. Four samples of Natal sweet orange on cleopatra mandarin (three from symptomatic trees and one from an asymptomatic tree), were collected in April 1992, in Colina, S.P., Brazil. Six other samples of Valencia sweet orange on Rangpur lime, were collected only from symptomatic trees in May, 1992 in Macaubal, S.P., Brazil. Samples were kept at 5 C before culture of the bacterium was attempted.

Culture media. Five media were prepared for the isolation and primary cultivation of the CVC-bacterium from twigs of sweet orange trees. Media CS20 and PD2 were prepared according to (1) and (4), respectively. Medium PW (3) was slightly modified in that $MgSO_4.7H_20$ 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; $MgSO_4.7H_2O$, 0.4 g; phenol red (0.2%), 10.0 ml; and agar, 12.0 g. Before pouring plates, 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%) were 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 and collected from symptomatic trees, and #5 from the asymptomatic tree) received in Bordeaux on April 21, 1992, were used for the culture assay on April 23, using 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, using 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 (each about 5-6 cm in length) were surface-sterilized in 1.06% sodium hypochlorite 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, using a loop was dropped onto duplicate plates (5 drops per plate) of each medium. The inoculated plates were kept in plastic bags and incubated at 30 C. Plates were observed for colony development at weekly intervals for a month with a binocular microscope.

Electron microscopy. Log-phase cells of isolate 8.1.b were used for electron microscopic observations. For negative straining, cell suspensions were prepared by suspending scraped 8-day-old cultures 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.

For ultrathin sections, blocks of colonies (1 × 2 mm) directly sectioned from an agar plate of an 8-day-old culture on PW were fixed with 2% glutaraldehyde and post-fixed with 1% osmium tetroxide for 2 hr each. They were then dehydrated in an ethanol series (50, 75, 95 and 100% for 30 min each). The agar blocks were embedded in Epon 812 mixture, and sectioned with an ultramicrotome (LKB ultrotome 3). Sections were stained with 1% uranyl acetate and lead citrate, and examined in a Philips CM 10 transmission electron microscope.

Inoculation of sweet orange seedlings. Twelve Hamlin sweet orange seedlings were mechanically inoculated, by stem injection, on June 4, 1992 and nine seedlings on June 17, 1992. Fifteen seedlings were injected with 50 to 250 µl of a 10-day-old broth culture of the CVC-bacterium in PW medium; six seedlings received equivalent amounts of uninoculated PW medium. On June 4, 1992 three additional seedlings were also inoculated by putting PW agar with CVC-colonies under the bark. This resulted in damaging the xylem tissues. On June 17, 1992 four additional seedlings were inoculated by vacuum infiltration. Of the latter, three received 500 µl to 1 ml of a 10-day-old culture; while one received equivalent amounts of PW medium. The multiplication of the bacterium in the seedlings was monitored by DAS-ELISA with a CVC-specific antiserum (2, 5). A first ELISA testing was done on August 26, 1992 with a mixture of two leaf midribs, one taken immediately above the inoculation point, the other taken at the top of the plant. A second ELISA testing was done on November 5, 1992 with two leaf midribs taken at the top of the seedling, far from the inoculation point.

RESULTS

Culture of the bacterium associated with CVC. In the first isolation attempt, four out of 12 symptomatic twigs yielded bacterial colonies on medium CVC1 and two out of 12 on medium CVC2; no colonies were obtained from asymptomatic healthy twigs (Table 1). 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 (Table 2). 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

TABLE 1
PRIMARY ISOLATION OF THE CVC-BACTERIUM

Sample no.	Twig no.	Symptomatic (S) or Asymptomatic (A) Trees	Media[a] CVC1	Media[a] CVC2
1	1	S	Cont.[b]	Cont.
	2	S	–[c]	–
	3	S	Cont.	Cont.
	4	S	+	–
3	1	S	–	–
	2	S	–	–
	3	S	–	–
	4	S	–	–
4	1	S	+	+
	2	S	+	+
	3	S	+	–
	4	S	Cont.	Cont.
5	1	A	–	–
	2	A	–	–
	3	A	–	–

[a]The incubation period was for 14 days.
[b]Cont. = contamination by fast growing saprophytes.
[c]+ and – = development and no development of colonies, respectively.

tissues showing CVC-symptoms will be designated as the CVC-bacterium.

Media CVC1 and CVC2 were suitable for the isolation of the CVC-bacterium. It took approximately two weeks before the colonies became visible under a binocular microscope on both media (Fig. 1 A and B). The recovery rate in medium CVC1 was approximately twice that on medium CVC2. The average number of colonies obtained from one loopful of tissue homogenate was 22.5 on medium CVC1 as compared to 14.5 on medium CVC2. These results suggest that the CVC-bacterium prefers medium CVC1 to medium CVC2. The only difference between these two is that the latter contains hemin chloride which seems to inhibit the bacterial growth under these conditions.

When three additional media were used in subsequent isolation attempts,

TABLE 2
PRIMARY ISOLATION OF THE CVC-BACTERIUM: COLONY DEVELOPMENT ON DIFFERENT MEDIA, AFTER 9 AND 25 DAYS OF INCUBATION

Sample no.	Twig no.	PW 9[a]	PD2 9	CS20 9	CS20 25	CVC1 9	CVC1 25	CVC2 9	CVC2 25
6	1	+[b]	+	+		+		+	
	2	+	+	+		+		+	
7	1	+	+	+		–	+	–	+
	2	+	+	+		–	+	–	+
8	1	+	+	–	+	–	+	–	+
	2	+	+	+		–	+	–	+
9	1	+	+	+		+		+	
	2	+	+	+		+		+	
10	1	+	+	–	+	–	+	–	+
	2	+	+	–	+	–	+	–	+
11	1	+	+	+		+		+	
	2	+	+	+		–		–	+

[a]Incubation was from May 25 to June 18, 1992, i.e. 25 days. Results were recorded on June 2 and June 18, i.e. 9 days and 25 days after incubation, respectively.
[b]+ and –: represent development and no development of colonies, respectively.

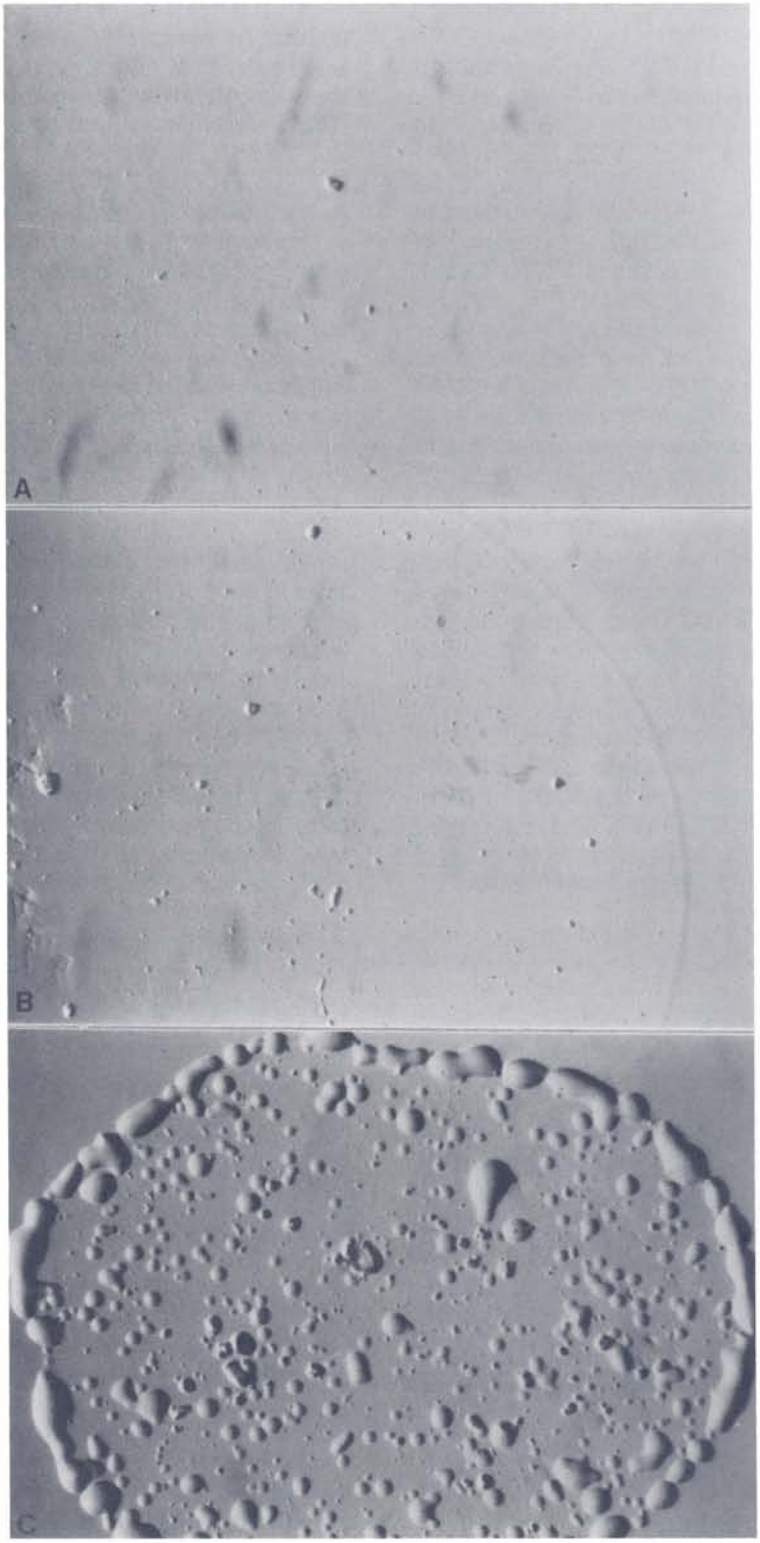

Fig. 1. Colonies of the CVC-bacterium obtained from a drop of tissue homogenate; A) on CVC2 medium after 12 days incubation (× 40); B) on CVC1 after 9 days incubation (× 40) and C) on PW after 9 days inoculation (× 16).

it was obvious that the CVC-bacterium preferred PW (Fig. 1 C), PD2 and CS20 to CVC1 and CVC2. The bacterial colonies became visible in 5 days on PW, 7-10 days on PD2 and CS20, and 9-14 days on CVC1 and CVC2. Hemin chloride did not seem to inhibit the growth of the bacterium when incorporated in PW, PD2 and CS20. The difference between PW and medium CVC2 is the two undefined components. PW contains trypticase peptone and phytone peptone (both purchased from BBL) whereas CVC2 has tryptone and Bacto-peptone (both from Difco). The trypticase peptone and phytone peptone seem to contain ingredients that promote faster growth of the bacterium.

Morphology of the CVC-bacterium. The morphology of the CVC-bacterium grown on PW agar plate is shown on Fig. 2. Cross sections through colonies reveal filamentous, rod shaped bacteria, 0.2-0.4 μm in diameter (Fig. 3 A). At higher magnification (Fig. 3 B), a rippled wall can be observed. After negative staining of the bacterium, the rippled surface of the wall is clearly seen (Fig. 3 C and D). The rod-shaped bacteria are 1.4 to 3 μm in length and appear to divide by binary fission (Fig. 3 C, arrows).

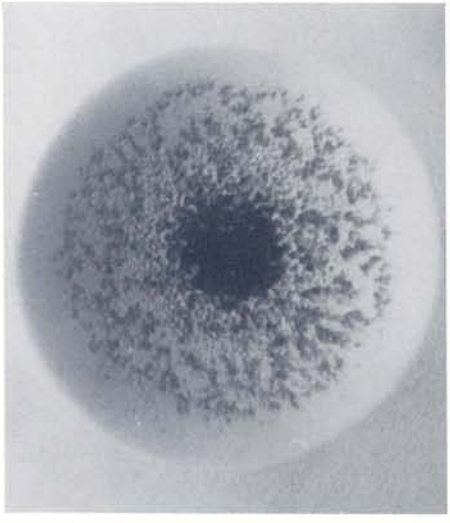

Fig. 2. Colony of the CVC-bacterium grown on PW medium (× 120).

Multiplication of the CVC bacterium in inoculated sweet orange seedlings. Five out of eight seedlings stem-inoculated with a culture of the CVC bacterium on June 4 gave a positive ELISA on August 26 (plants 1, 2, 5, 6, and 7) (Table 3). One additional seedling (plant 8) gave a positive ELISA on November 5. At that time, even though no symptoms could be observed on the positive seedlings, the bacterium was systemic in the plant. Indeed, at that date, the leaves used for ELISA were taken at the top growing point of the seedling, far from the inoculation site. Optical densities (OD) were between 0.3 and >2 indicating a good concentration of the bacterium in the seedling. Two (plants 17 and 19) out of seven seedlings stem-inoculated on June 17 gave positive ELISA reactions in August and November. None of the seedlings inoculated with bacterial colonies were positive in August; however, in November, two (plants 9a and 10) out of three gave positive results in ELISA with high ODs. The vacuum-infiltrated seedlings did not grow well compared to seedlings inoculated by other techniques. One was already dead in November and the others died later on. No positive ELISA reactions were obtained with these plants. As expected, seedlings inoculated with PW medium gave negative ELISA results.

At the time when the seedlings were tested, they were symptomless. However, a chlorotic variegation started to develop on a few leaves of certain ELISA positive seedlings by mid November.

CONCLUSIONS

The bacterium found in the xylem of CVC-affected sweet orange trees has been cultured consistently in several media previously devised to culture *Xylella fastidiosa*. The cultured bacterium had the same size and morphology as the bacterium present in xylem tissues. The fastest growth was obtained on modified PW medium. Inoculation of the cultured bacterium into healthy sweet orange seedlings resulted in a high con-

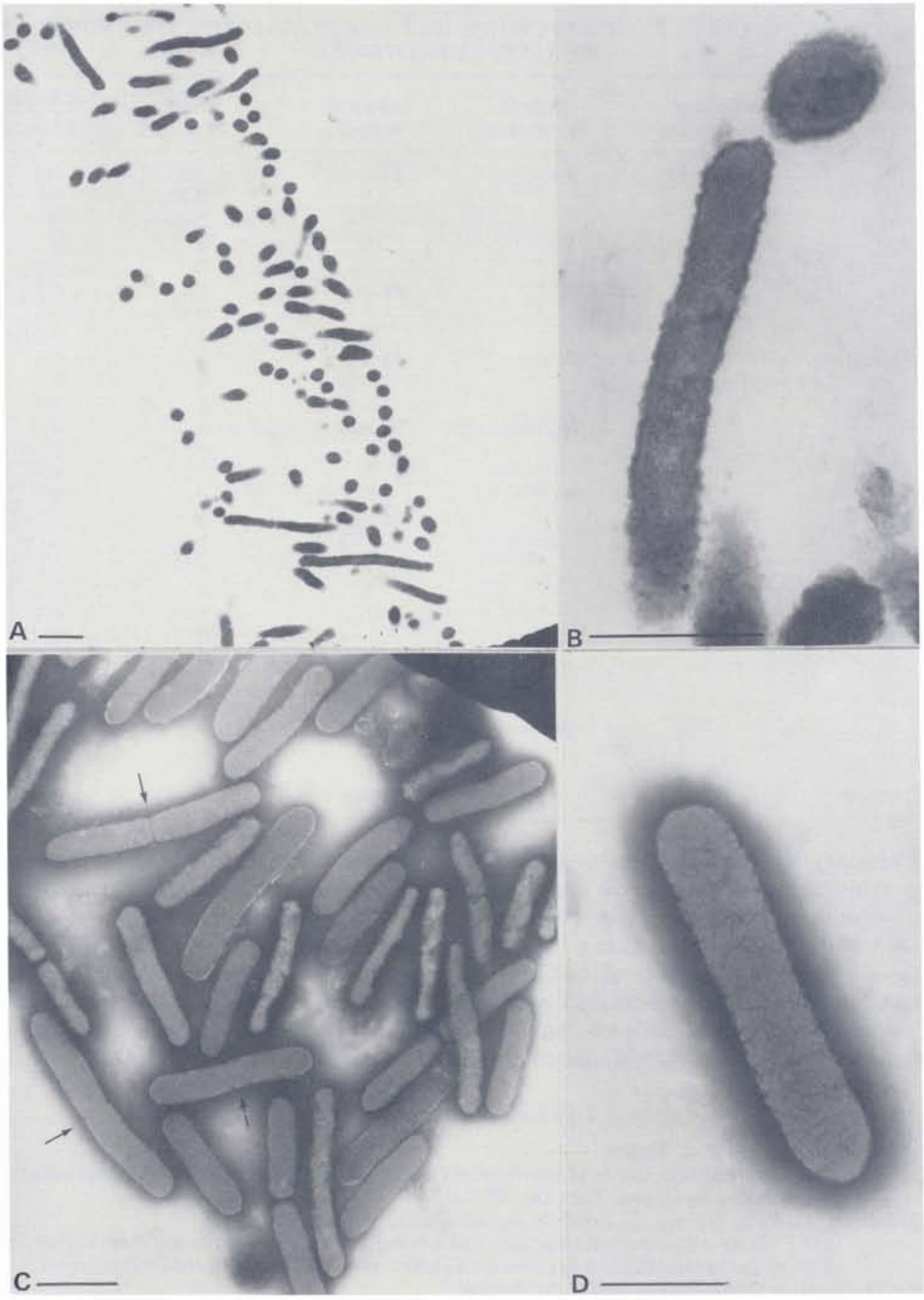

Fig. 3. Electron micrographs of cultured cells of the CVC-bacterium. The bars represent 1 μm (A, C), 0.5 μm (B, D); A, B) cross section through a colony on PW medium; C, D) negative staining, arrows indicate bacteria dividing by binary fission. Note wavy outer wall layer.

centration of the bacterium in the plant 3 months after inoculation. It became systemic after 5 months and symptoms started to develop soon thereafter.

The symptoms observed mid-November continued to develop and extended to additional leaves in December-January. They were conspicuous in

TABLE 3
DETECTION OF CVC IN PLANTS MECHANICALLY INOCULATED WITH THE CVC STRAIN OF *XYLELLA FASTIDIOSA*

Plant no.	Date of inoculation	Mode of inoculation	Inoculated sample	ELISA (OD after 30 min) 26 Aug 92	5 Nov 92
1	4 Jun 92	Injection	#4-1	>2	>2
2	"	"	"	0.395	0.3
3	"	"	"	0	0
4	"	"	"	0	0
5	"	"	"	0.6	>2
6	"	"	#5-2	0.7	0.7
7	"	"	"	0.45	0.5
8	"	"	"	0	0.250
9a	"	Colony	#4-2	0	0.6
9b	"	"	"	0	0
10	"	"	"	0	0.5
11	"	Injection	Medium	0	0
12	"	"	"	0	0
13	"	"	"	0	0
14	"	"	"	0	0
15	17 Jun 92	"	#4-1a	0	0.130
16	"	"	"	0	0.110
17	"	"	"	0.27	>2
18	"	"	"	0	0
19	"	"	"	0.1	0.3
20	"	"	"	0	0
21	"	"	"	0	0
22	"	"	Medium	0	0
23	"	"	Medium	0	0
24	"	Vacuum	#4-1a	0	0
25	"	"	#4-1a	0	0
26	"	"	#4-1a	0	dead
27	"	"	Medium	0	0

February-March, i.e. 9 to 10 months post inoculation. We have now observed a faint gumming associated with the chlorotic lesions on the lower face of the leaves (5). Reisolation of the CVC-bacterium from two inoculated seedlings (plants 5 and 17, Table 3), on PW medium was successful in January 1993 with the two plants. Bacterial colonies which reacted with the CVC-antiserum were obtained after five days on this medium. Therefore, fulfillment of Koch's postulates had been achieved. We conclude that the CVC bacterium is the etiological agent of Citrus Variegated Chlorosis.

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