Citrus Variegated Chlorosis: Serological Detection of *Xylella fastidiosa*, The Bacterium Associated With the Disease

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

ABSTRACT. A bacterium resembling *Xylella fastidiosa* has been cultured from citrus variegated chlorosis-affected citrus plants. Polyclonal antibodies have been obtained against the cultured bacterium and used in DAS-ELISA for its detection and characterization. The bacterium is closely related to other *X. fastidiosa* strains and can be detected in infected trees, both on symptomatic and asymptomatic leaves. The detection of the bacterium by a fast immunoprinting assay is described.

We have cultured the xylem-restricted bacterium causing citrus variegated chlorosis (CVC) in cell-free media (1, 2). Here we describe the production of an antiserum against the cultivated bacterium, and its use to characterize the CVC-bacterium as a strain of *Xylella fastidiosa*. The serum has also been used for the detection of the CVC-bacterium in citrus tissues by double sandwich antibody-enzyme linked immunosorbent assay (DAS-ELISA) and immunoprinting. The results show that the CVC-bacterium can be detected in citrus trees before symptom development.

MATERIAL AND METHODS

Production of antiserum. A 500 ml broth culture of isolate 8.1.b (1) of the CVC-bacterium in medium PW (4) was centrifuged at 20,000 × g for 30 min. The resulting pellet was 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 from the ear vein, one week after the last injection. The serum was collected, centrifuged at 1,500 × 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. One mg of conjugate for DAS-ELISA was obtained by labelling with alkaline phosphatase (AP).

Production of monoclonal antibodies. Monoclonal antibodies (MA) were produced following standard protocols after immunization of a Balb C mouse with three intraperitoneal injections of 35 µg of CVC-proteins each and one intravenous injection with 60 µg of CVC-proteins.

Double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA). DAS-ELISA was done according to Clark and Adams (2). 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 hr. After three washes with PBS-tween buffer, the antigen was added at a protein concentration of 30 µg/ml and the ELISA plates were incubated overnight at 4 C. The AP-conjugate at the indicated dilutions was added after three washes with PBS-tween buffer, and the plates were incubated at 37 C for 4 hr 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 hr at 37 C) and with a 2,000 fold dilution of the AP-conjugate (4 hr 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 cheese cloth, and 150 μl of filtrate were placed in each well of the ELISA plate overnight at 4 C.

Eight type strains of *X. fastidiosa* obtained from the American Type Culture Collection (ATCC) and five Georgian strains originally isolated from grapevines showing 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.

Immunoprinting. Cross sections were cut through leaf midribs or petioles from healthy or CVC-affected citrus trees with a scalpel and the sectioned parts were immediately blotted onto nitrocellulose membranes. The membranes were allowed to dry, then were saturated with a solution of 1% BSA. The membranes were incubated with a 2000 fold dilution of the AP-conjugate (see above). The blots were revealed with a solution of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP-NBT).

RESULTS

Detection of the CVC-bacterium by ELISA.

Conditions for the DAS-ELISA and sensitivity of the assay for the detection of cultured cells of the CVC bacterium. The conditions for the ELISA were evaluated using different concentrations of Ig for coating the ELISA plate and different dilutions of the AP-conjugate. 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 to 0.6). At lower concentrations of Ig for coating, the OD of the control wells was generally below 0.1 and high OD were obtained with the CVC-bacterium. From these results, we chose a concentration of 5 μg/ml for coating the ELISA plate and a 2000 fold dilution of the AP-conjugate.

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 when a concentration of the homologous antigen of only 1.2 μg proteins/ml was used. The reading was still significant (0.238) when the antigen concentration was as low as 0.035 μg/ml. The other isolates from CVC-infected material also reacted positively. No reaction was noticed with *E. 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 of the CVC-bacterium was able to detect 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 (Table 1). Midribs from healthy sweet orange trees, from greening-affected trees grown in the Bordeaux greenhouse, and from asymptomatic trees in CVC-affected orchards, or orchards without CVC, gave OD readings close to zero.

The highly positive ELISA reactions obtained in Bordeaux with shipped plant material were readily duplicated in 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, irrespective of area (Barretos, Cocal, Taquaritinga, Catigua), sweet orange variety (Pera, Natal, Valencia,

TABLE 1
DETECTION OF THE CVC-BACTERIUM IN HOMOGENATES OF SWEET ORANGE LEAF MIDRIBS

Sweet Orange Cultivar	Disease Affecting Leaves	Origin	DAS ELISA[a] (OD 405 nm)
Hamlin	None	Bordeaux greenhouse	0.009
Hamlin	Greening	Bordeaux greenhouse	0.010
Valencia	CVC	Macaubal SP	>2
Valencia	CVC	Macaubal SP	>2
Valencia	CVC	Taquaritinga SP	>2

[a]Antiserum prepared against a cultured isolate of the CVC-bacterium.

Hamlin) or rootstock. ELISA was negative with leaf midribs from symptomless trees in CVC-affected orchards or from trees, 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 and gave negative reactions.

A blind test for CVC was done by ELISA of midribs from 24 samples shipped to Bordeaux without indication of disease status. All ELISA positive samples come from symptomatic trees whereas all negative reactions were given by trees from CVC-free orchards whether they were in CVC-affected or in CVC-free regions (Table 2). Positive reactions were even obtained with midribs of symptomless leaves collected from trees with CVC-affected fruits (sample 15).

Leaves from Tabay (Argentina) with symptoms of Pecosita disease, a disease very similar to CVC, were also tested (Table 2, samples 16-1 to 20). They gave strongly positive ELISA reactions (OD comprised between 0.2 and >2), indicating that the disease is present not only in Brazil but also in Argentina, and that CVC and Pecosita disease are similar.

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). These strains belong to group I of the *X. fastidiosa* strains, as described by Purcell (5). In group II, the ragweed stunt strain gave a strong reaction, but the periwinkle, mulberry and oak strains did not react (Table 3).

Detection of the CVC-bacterium by immunoprinting

Immunodetection of the CVC-bacterium after printing of leaf midribs or petioles from healthy or CVC-infected citrus plants has been carried out. A purple blue coloration was clearly seen in the xylem on prints from infected midribs but not in that from healthy midribs, showing that this technique can be used for quick detection of the bacterium.

Monoclonal antibodies

A total number of 819 hybridomas were obtained after fusion of the CVC-immunized mouse splenocytes with myeloma cells. Seven of them were shown to produce CVC-specific MAs by ELISA. The specificity of the MAs versus other *Xylella fastidiosa* strains is under study.

CONCLUSION

Polyclonal and monoclonal antibodies have been produced against isolate 8.1.b of the CVC-bacterium. Using the polyclonal antibodies, detection of the CVC bacterium has been achieved by ELISA with a very good sensitivity. Detection of the CVC-bacterium can also be achieved with a quick immuno-

TABLE 2
BLIND TESTING FOR CVC BY ELISA

Sample Number	Origin	Varietal Combination	CVC Symptoms					ELISA[a]
			Orchard	Tree	Branch	Leaves	Fruits	
1	Macaubal	Pera/Rangpur	+	+	+	−	+	0.325
2	"	"	+	+	+	−	+	>2
3	"	"	+	+	+	+	+	>2
4	"	"	+	+	−	−	+	>2
7	Moji Guaçu	Valencia/Rangpur	−	−	−	−	−	0.001
8	"	"	−	−	−	−	−	0.003
9	"	"	−	−	−	−	−	0
10	Macaubal	Wash. N.[b]/Rangpur	+	−	−	−	−	0
11	"	Natal/Cleopatra	+	−	−	−	+	0
12-1	Taquaritinga	Pera/Rangpur	+	+	+	+	+	>2
12-2	"	"	+	+	+	−	+	>2
12-3	"	"	+	+	−	−	+	0.73
15	Colina	Natal/Cleopatra	+	−	−	−	+	>2
16-1	Tabay (Arg)	Val. Late[c]/Rangpur	+	+	+	+	−	1.4
16-2	"	"	+	+	+	−	−	0.6
16-3	"	"	+	+	−	−	−	0.218
18	"	Val. Late/Troyer	++	++	+	+	++	>2
20	"	Val. Seedless/Rangpur	+	−	−	−	−	0.005
21	Colina	Natal/Rangpur	−	−	−	−	−	0.010
22	"	"	−	−	−	−	−	0.008
23	"	Valencia/Troyer	−	−	−	−	−	0
24	"	"	−	−	−	−	−	0.001

[a]ELISA conducted with leaf midrib homogenates. Antiserum prepared against isolate 8.1.b. of the CVC-bacterium.
[b]Wash. N. = Washington Navel.
[c]Val. late = Valencia late.

TABLE 3
SEROLOGICAL RELATEDNESS BETWEEN THE CVC-BACTERIUM AND STRAINS OF *XYLELLA FASTIDIOSA*

Antigens Tested	ATCC Number	OD[a] at 405 nm 15 min	OD[a] at 405 nm 30 min
CVC-bacterium, isolate 8.1.b	—	0.860	>2
Xylella fastidiosa from:			
Mulberry leaf scorch	35868	0.022	0.057
Mulberry leaf scorch	35869	0	0.032
Oak leaf scorch	35874	0.014	0.045
Ragweed stunt	35876	0.701	>2
Periwinkle wilt	35878	0	0.07
Almond leaf scorch	35870	0.743	>2
Pierce disease of grape	35879	0.852	>2
Pierce disease of grape	35881	0.815	>2
Pierce disease of grape:			
Georgia isolate Chateau 3C	—	0.613	>2
Georgia isolate 112.V1	—	0.814	>2
Georgia isolate 116.V6	—	0.737	>2
Georgia isolate 116.V11	—	0.601	>2
Georgia isolate MS7	—	0.654	>2
E. coli	—	0.059	0.083

[a]ELISA conducted using antiserum prepared against isolate 8.1.b of the CVC bacterium.

printing assay. The use of polyclonal antibodies has shown that the CVC-bacterium was a strain of *Xylella fastidiosa* as already suggested by its xylem location, morphology and growth requirements (1, 6).

We were also able to demonstrate that *X. fastidiosa* is also associated with Pecosita disease in Argentina.

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