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Citrus Variegated Chlorosis: Detection of *Xylella fastidiosa* in Symptomless Trees

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ABSTRACT. *Xylella fastidiosa* has been associated with citrus variegated chlorosis (CVC) by electron microscopy and serology using antisera to isolates of *X. fastidiosa* other than citrus. Antiserum has now been developed against *X. fastidiosa* isolated from CVC-affected citrus. This antiserum has been used to develop a sensitive, reliable serological assay based on a modification of dot immunobinding assays previously reported, coupled with western blotting, to detect *X. fastidiosa* in CVC affected trees. The procedure detects *X. fastidiosa* even in CVC-inoculated trees even before they develop symptoms, in some non-symptomatic trees in groves affected by CVC, and in healthy appearing sectors of trees which are showing CVC symptoms in other sectors. Bacteria were not detected in many healthy appearing trees from groves in non-CVC affected areas or in healthy greenhouse plants. This sensitive serological assay was developed to better enable screening of symptomless CVC-affected nursery plants and budwood source trees to reduce the possible spread of CVC via infected nursery materials.

Index words. Xylem-limited bacteria, dot immunobinding assay, western blotting assay.

A new citrus disease was observed around 1987 in São Paulo and Minas Gerais States, Brazil and was called citrus variegated chlorosis - CVC (6, 11, 12). This disease affects all the commercial orange varieties grown in Brazil, and has been spreading in nurseries and commercial orchards (6). A survey made by FUNDECITRUS (Brazilian Citrus Foundation) in 1992, found that 84% of the whole citrus plantation area is affected by this disease, with over 2 million trees already destroyed or rendered nonproductive by CVC (13).

A xylem-inhabiting bacterium with a morphology similar to *Xylella fastidiosa* has been associated with CVC by electron microscopy (4, 12). The disease symptoms are ameliorated by application of oxytetracycline antibiotics (3) indicating the disease is caused by an agent susceptible to this antibiotic. Strains of *X. fastidiosa* have been isolated from symptomatic twig and leaf tissue from CVC-affected trees (2, 7, 8, 10) and polyclonal antibodies have been produced against these isolates *in vitro* (2, 10). A dot immunobinding assay (DIBA) was developed to detect the presence of *X. fastidiosa* in CVC-affected trees, using initially antibodies made against isolates of *X. fastidiosa* from other hosts (1, 6, 8), and subsequently antibodies made against

the *X. fastidiosa* isolates cultured from CVC affected trees (2, 10). The use of DIBA assays indicate a constant association of *X. fastidiosa* with CVC-affected trees. More recently, Koch's postulates have been fulfilled with CVC in the quarantine greenhouse, USDA Fruit Laboratory, Beltsville, MD, indicating the *X. fastidiosa* isolated from CVC-affected plants is the etiological agent of CVC (10, J. Hartung, personal communication).

In this paper we describe a more sensitive serological assay which can be used to detect *X. fastidiosa* in asymptomatic CVC-infected plants which should enable screening of budwood source trees and nursery material thereby lessening the chances of passage of CVC by use of infected propagating materials.

MATERIALS AND METHODS

Plant material. Leaf midribs were collected from symptomatic and asymptomatic sweet orange trees in the field from the northern part of São Paulo State, Brazil. Material was collected from 20 trees each of Valencia, Pera, and Bahia-Navel sweet orange varieties which were being used as budwood source trees from two different foundation blocks. Leaf midribs

were collected from ten symptomatic and ten asymptomatic nursery plants from different commercial nurseries in the same area. Samples were collected from healthy sweet orange plants in the greenhouse of the Instituto Biológico in São Paulo for use as healthy controls. All leaf midribs were dried in sealed tubes containing silica gel before being used for serological assay.

In January 1992 an experiment to fulfill Koch's postulates was established in the quarantine greenhouse, USDA Fruit Laboratory, Beltsville, MD, USA in which Valencia and Madame Vinous sweet orange and rough lemon plants were inoculated with axenic cultures of *X. fastidiosa* isolated from CVC affected fruit peduncles and leaf petioles (2, 7, 10). Ten plants were inoculated by pin prick inoculation through 50 μ l droplets of inoculum containing 10^8 cells/ml in PBS buffer (0.01 M KPO_4 , 0.15 M NaCl, pH 7.4) plus 0.1% Tween-20 (PBST). An equal number of control inoculations were made using PBST without bacteria. Additional inoculations were made to the same plants at about 3-month intervals by placing a bark flap in the inoculum, and letting the plant imbibe the inoculum (or PBST buffer only) for 2 hr. Midribs from these plants were collected at intervals and used for serological assays.

Antisera. Polyclonal antiserum (UF 26) was made using the CVC isolate of *X. fastidiosa* isolated from CVC-affected tissue using the protocol previously described (2).

DIBA. The dot-immunobinding assay (DIBA) as originally described by Lee *et al.* (5, 7) for detection of *X. fastidiosa* in CVC-affected trees was modified to improve the sensitivity. Dried midrib tissue was chopped in extraction buffer (0.25 M Tris, 8% SDS, 40% glycerol, 4% 2-mercaptoethanol, and 0.5% bromophenol blue) and left overnight at room temperature. The preparation was then boiled in a water bath for 5 min. After cooling to room temperature, 2 μ l were spotted onto a nitrocellulose membrane, and dried for

5 min. The membrane was covered with water in a tray and microwaved for 1 min. After cooling, the membrane was blocked using 5% nonfat dry milk in TBS (0.05 M Tris, 0.15 M NaCl, pH 8.0) with 0.1% Tween-20 (TBST) for 30 min. After blocking, the membrane was rinsed three times with TBST, then incubated in UF 26 IgG (1 μ g/ml), which had been purified from whole serum using a Protein A affinity column, in conjugate buffer (TBST plus 0.5% BSA) for 6 hr to overnight at room temperature. After rinsing three times in TBST, the secondary antibody conjugate (goat anti-rabbit IgG with alkaline phosphatase) was added in conjugate buffer at the dilution recommended by the supplier (Sigma), usually 1/7000 dilution. After incubating from 6 hr to overnight, the membrane was rinsed three times in TBST. The membrane was then incubated in the nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrate as previously described by Lee *et al.* (7).

Western blot assay. A 5 μ l aliquot of the same extract as used for DIBA was used for western blot assay. The proteins in the sample were separated by SDS-PAGE using 12% gels, then transferred to nitrocellulose using a semi-dry electroblot apparatus. After the proteins had been transferred to the nitrocellulose membrane, the membrane was developed as previously described for DIBA.

RESULTS AND DISCUSSION

The DIBA as described in this paper has an advantage over the previous procedure (7) in that green color from the plant extracts does not remain on the nitrocellulose membrane to cause a problem in detecting weakly positive samples. Table 1 summarizes the relative reactivity of antibodies made against Pierce's disease (PD), plum leaf scald (PLS) and CVC isolates of *X. fastidiosa* with several antigens from various isolates of *X. fastidiosa* and against extracts from CVC and healthy citrus midribs. Using cultures of CVC isolates of *X. fastidiosa*, the DIBA permits de-

TABLE 1
COMPARISON OF THE DOT IMMUNOBINDING ASSAYS (DIBA) USING ANTISERA TO DIFFERENT ISOLATES OF *XYLELLA FASTIDIOSA* TO DETECT *X. FASTIDIOSA* FROM CULTURES AND FROM PLANT TISSUES

Sample	Concentration (cells/ml)	DIBA ^z		
		PD	PLS	CVC
<i>X. fastidiosa</i> CVC isolate	10 ⁷	1	3	5
	10 ⁶	0	1	4
	10 ⁵	0	0	2
	10 ⁴	0	0	1
PD-LH isolate	10 ⁸	2	3	1
	10 ⁷	1	1	0
	10 ⁶	0	0	0
PP-5 isolate	10 ⁸	3	5	4
	10 ⁷	1	4	3
	10 ⁶	0	2	2
	10 ⁵	0	1	0
	10 ⁴	0	0	0
PD-Tulare isolate	10 ⁸	4	3	1
PD-CR	10 ⁸	3	3	1
<i>Xanthomonas campestris</i> pv. <i>vesicatoria</i>	10 ⁸	0	0	0
<i>Corynebacterium</i> <i>michiganense</i>	10 ⁸	0	0	0
Healthy Valencia	midribs	0	0	0
Healthy Madame Vinous	midribs	0	0	0
CVC-infected	midribs	3	4	5

^zDIBA was run as described using IgG purified from antiserum made against Pierce's disease of grapevines (PD), plum leaf scald (PLS) of plum, or citrus variegated chlorosis (CVC) isolates of *X. fastidiosa*. The DIBA results were rated visually on a scale whereby 5 = very intense reaction to 1 = faintest detectable reaction which can be rated as a positive and 0 = no reaction.

tection of 10⁴ cells/ml (Table 1). This is a 100-fold increase in sensitivity as compared to ELISA assays with *X. fastidiosa* (7). The antiserum prepared against the CVC isolate of *X. fastidiosa* reacts strongly with CVC antigen, moderately strongly with phony peach isolates, and least strongly with Pierce's disease isolates of *X. fastidiosa* in DIBA assays. The DIBA assay described here permits the same extract used for DIBA to be used also for western blot analysis, further verifying the presence of *X. fastidiosa* by the unique "fingerprint" of

proteins reacting with the polyclonal antibodies. The "fingerprint" of antigenic proteins from the CVC bacterium is illustrated in Fig. 1.

No positive results were observed in the samples collected from the budwood source trees from two different foundation plantings. *X. fastidiosa* was detected in leaves collected from the asymptomatic sectors of trees just developing CVC symptoms. Positive results were consistently obtained when using leaf samples collected from symptomatic trees. Ten of ten-yr-old

Fig. 1. Western blot of SDS-PAGE gel developed using UF 26 polyclonal antiserum. Lane 1 contains an extract from a Pera sweet orange tree from Brazil which was showing CVC symptoms, lane 2 contains the prestained protein standards which are used to monitor transfer of the proteins from the SDS-PAGE gel to the nitrocellulose membrane. Lanes 3-12 contain extracts from plants inoculated with the *Xylella fastidiosa* culture obtained from CVC infected material, coded plants A through J, respectively. Lane 7 is from a sweet orange plant (coded plant E) which was inoculated with *X. fastidiosa*, but was not yet showing symptoms. This plant has subsequently developed symptoms characteristic of CVC and bacteria have been reisolated from this plant (10). Lane 13 contains a CVC positive control and lane 14 contains a Pierce's disease positive control. Both positive controls are from infected field material. Lane 15 is a negative control from healthy tissue.

nursery plants showing CVC symptoms and six out of ten plants of the asymptomatic nursery plants were positive for presence of *X. fastidiosa* in the DIBA and western blot analysis.

The ten plants inoculated with the CVC isolate of *X. fastidiosa* and the ten control plants inoculated with PBST in Beltsville were assayed in May 1992, August 1992, October 1992, and January 1993. All plants were negative when assayed in May 1992, but plant number 5, which was inoculated with bacteria, was positive on all other assay dates. The results from the August 1992 assay are shown in Fig. 1. Plant number 5 began showing symptoms of CVC in the same month, and a *X. fastidiosa* was re-isolated in January 1993 and Koch's Postulates completed (10). Thus, the use of

DIBA indicated the presence of *X. fastidiosa* in this plant 5 months before the plant developed symptoms.

The DIBA and western blot assays for detection of *X. fastidiosa* in infected plant tissue as described here permit detection of the bacteria in affected trees before the onset of visual symptoms of CVC. This assay should permit screening of nursery material and budwood source tissue to reduce the chance of passage of *X. fastidiosa* through nursery propagations by the use of CVC infected budwood. Further work is being done to detect the presence of *X. fastidiosa* in the sharpshooter vectors and to quickly assess the serological relatedness of unknown isolates of *X. fastidiosa* to determine if they are more closely related to Pierce's disease or CVC.

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LITERATURE CITED

1. Beretta, M. J. G., E. E. Bach, R. F. Lee and K. S. Derrick
1991. Serological comparison of citrus blight from Florida with decline and citrus variegated chlorosis in Brazil. *Summa Phytopathologica* 17: 51 (Abstr.).
2. Beretta, M. J. G., R. F. Lee, K. S. Derrick, C. L. Davis and G. A. Barthe
1992. Culture and serology of a *Xylella fastidiosa* associated with citrus variegated chlorosis in Brazil. *In: Proc. 7th Int. Citrus Congress, Italy* (in press).
3. Beretta, M. J. G., R. F. Lee, K. S. Derrick, J. R. Santiago Oliveira, J. Thomé Neto, A. L. Rodrigues, and A. R. R. Teixeira
1992. Remission of citrus variegated chlorosis (CVC) symptoms by treatment with oxytetracycline. *Summa Phytopathol.* 18 (1): 5 (abstr.).
4. Chagas, C. M., V. Rossetti and M. J. G. Beretta
1992. Electron microscopy studies of xylem-limited bacterium in sweet orange affected with citrus variegated chlorosis disease in Brazil. *J. Phytopathol.* 134(4): 306-312.
5. Lee, R. F., M. J. G. Beretta and K. S. Derrick
1991. Development of a dot immunobinding assay for citrus variegated chlorosis disease in Brazil. *Fitopatologia* 26: 46-47 (Abstr.).
6. Lee, R. F., K. S. Derrick, M. J. G. Beretta, C. M. Chagas and V. Rossetti
1991. Citrus variegated chlorosis: a new destructive disease of citrus in Brazil. *Citrus Industry*, 72(10): 12-13, 15.
7. Lee, R. F., M. J. G. Beretta, K. S. Derrick and M. E. Hooker
1992. Development of a serological assay for citrus variegated chlorosis - a new disease of citrus in Brazil. *Proc. Fla. State Hort. Soc.* 105: 32-34.
8. Lee, R. F., M. J. G. Beretta, K. S. Derrick, M. E. Hooker, C. L. Davis, and R. H. Brlansky
1994. Culture of *Xylella fastidiosa* from citrus trees affected with citrus variegated chlorosis in Brazil. *Phytopathology* 84 (abstr.) (in press).
9. Lee, R. F., M. J. G. Beretta, K. S. Derrick, V. Rossetti and C. Chagas
1992. *Xylella fastidiosa* associated with citrus variegated chlorosis disease in Brazil. *Summa Phytopathol.* 18(1): 57 (abstr.).
10. Lee, R. F., M. J. G. Beretta, J. Hartung, M. E. Hooker and K. S. Derrick
1993. Citrus variegated chlorosis: confirmation of a *Xylella fastidiosa* as the causal agent. *Summa Phytopathol.* 19: 123-125.
11. Rossetti, V., M. Garnier, M. J. G. Beretta, A. R. R. Teixeira, J. A. Quaggio, O. C. Battaglia, M. P. Gomes, J. D. De Negri and J. M. Bove
1990. Resultados preliminares de estudos sobre uma nova anormalidade dos citros observada nos Estados de S. Paulo e Minas Gerais. *Summa Phytopathol.* 16: 13 (abstr.).
12. Rossetti, V., M. Garnier, J. M. Bove, M. J. G. Beretta, A. R. R. Teixeira, J. A. Quaggio and J. D. De Negri
1990. Presence de bacteries dans le xyleme d'orangers atteints de chlorose varieguee une nouvelle maladie des agrumes au Bresil. *C. R. Acad. Sci. Paris* 310(Serie III): 345-349.
13. Tubellis, A.
1992. Difusão da clorose em pomares de São Paulo e Minas Gerais (Levantamento realizado pela FUNDECITRUS). *Informativo Coopercitrus* 72: 24-30.