Detection of the Sugarcane Leaf Scald Pathogen, *Xanthomonas albilineans*, Using Tissue Blot Immunoassay, ELISA, and Isolation Techniques

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ABSTRACT

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Tissue blot immunoassay, ELISA, and isolation techniques were compared for detection of *Xanthomonas albilineans* in sugarcane tissues at different positions along the stalk. *X. albilineans* was detected in the highest frequency in samples from mature plants at the internode superior to 62.5% of the total internodes. This tissue position had the highest frequency of detection and the highest optical density readings with ELISA for both symptomatic and asymptomatic stalks. The number of infected vascular bundles was highest in samples taken from the upper half of symptomatic stalks. The frequency of detection from symptomatic stalks was 67.0, 90.4, and 98.9% for isolation, ELISA, and tissue blot immunoassay techniques, respectively. *X. albilineans* was detected by the same three techniques, respectively, in 26.6, 6.3, and 16.2% of the asymptomatic stalks taken from the same plots. The tissue blot immunoassay technique was 98.9% effective in detecting *X. albilineans* in symptomatic stalks, and both that assay and isolation techniques would be most useful in detecting latent infections in asymptomatic stalks.

Leaf scald disease of sugarcane, caused by *Xanthomonas albilineans* (Ashby) Dowson, is found in at least 44 countries where sugarcane (interspecific hybrids of *Saccharum* spp.) is grown commercially (12). The disease is economically important in many of these locations. Leaf scald symptoms include one or more of the following: pencil-line leaf streaks, chlorotic leaf streaks with or without necrosis, profuse side shoot development on mature stalks, and internal reddish discoloration of vascular bundles at the nodes and at the juncture of the lateral side shoot and the stalk (12,14). However, plants infected with *X. albilineans* may not show any symptoms (12), making it difficult to accurately diagnose the disease or determine disease incidence. Because seed pieces can be cut and planted from latently infected plants, it is difficult to restrict the movement of the pathogen either in quarantine or in commercial operations. Furthermore, latent infections complicate disease screening.

Primary control of leaf scald relies on planting resistant cultivars. To a lesser extent, however, control is achieved by disease-free seed cane nursery programs. Sugarcane industries that have disease-free seed cane programs generally also have breeding programs to develop resistant cultivars. The seed cane nursery program relies on heat therapy, sanitation, and a rigid monitoring system to ensure disease-free seed cane. Monitoring seed cane is based on symptom expression and is hindered by the lack of reliable means to detect latent infection.

Bacterial plant pathogens have been detected using serological methods (1-4,7-11,13,15-17), including a number of plant pathogens belonging to the genus *Xanthomonas* (1,2,4). These methods are accurate and rapid. The leaf scald pathogen, *X. albilineans*, has been detected by means of enzyme-linked immunosorbent assay (ELISA) (3,8,10, 15,16), immunofluorescence (3,9,13,15), latex agglutination (3), and tissue blot enzyme immunoassay (TBIA) (6) techniques. Besides various polyclonal antisera, there are monoclonal antibodies (MAb's) specific for the genus *Xanthomonas* (1), for *X. albilineans* (16), and for each of the three serovars of *X. albilineans* (A. M. Alvarez, *personal communication*).

Our objectives were: 1) to determine the best tissue position to sample mature sugarcane stalks for detection of *X. albilineans* and 2) to compare ELISA, TBIA, and pathogen isolation for detection of *X. albilineans* in symptomatic and asymptomatic stalks.

MATERIALS AND METHODS

Sampling. Plant materials naturally infected with the leaf scald bacterium were obtained from mature plants 10-12 mo old from research plots of the USDA-ARS Sugarcane Field Station at Canal Point, Florida. The presence or absence of leaf scald symptoms on each stalk was noted at the time of collection. To determine the optimum location on the stalk for sampling, 60 stalks—28 (46.7%) symptomatic and 32 (53.3%) asymptomatic—were sampled. Samples were taken at internode positions 12.5, 37.5, 62.5, and 87.5% up from the base of the stalk, referred to as positions 1, 2, 3, and 4, respectively. A spatial measurement was not used, since the internodes on a sugarcane stalk vary in length. For comparison of the different detection techniques, 316 stalk samples—94 (29.7%) symptomatic and 222 (70.3%) asymptomatic—were assayed by all three techniques.

ELISA. The same primary and secondary antibodies (at 1:1,000 dilutions) were used in both ELISA and TBIA. The primary antibody was the X1 MAb specific for the genus *Xanthomonas* (1) that was either purchased from Adgia, Inc. (Elkhart, IN) or received directly from A. M. Alvarez (Department of Plant Pathology, University of Hawaii, Honolulu). The secondary antibody was a commercial goat antimouse-alkaline phosphatase antibody conjugate (Sigma, St. Louis, MO).

An indirect ELISA was conducted using 96-well polystyrene microtiter plates (Immulon II, Dynatech Laboratories, Alexandria, VA) with methods described previously (8). Vascular sap was obtained by centrifuging 1.6 × 5.0 cm cores of stalk tissue in 50-ml centrifuge tubes at 1,800 *g* for 10 min. The vascular sap was used immediately or stored at −20 C up to 14 days.

For the ELISA tests, the vascular sap was vortexed and a 400-µl aliquot was placed in a 1.5-ml microfuge tube and centrifuged at 8,500 *g* for 10 min. The supernatant was discarded and the pellet was resuspended in 400 µl of 0.05 M carbonate-bicarbonate buffer (pH 9.6). Bacterial suspensions from cultures were used in certain tests to serve as controls. A 100-µl aliquot of the suspension was placed in the well of a polystyrene microtiter plate and dried overnight at 35 C. The wells were blocked with 200 µl of 5.0% skim milk in 0.01 M phosphate-buffered saline with 0.05% Tween (PBST, pH 7.4) for 30 min. After this step and between all subsequent steps,

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the plates were washed three times with PBST. After the washing, 100 μl per well of the X1 MAb in PBST plus 2.5% skim milk was incubated for 1 hr at 30 C. After incubation with the primary antibody, 100 μl per well of secondary antibody was added and incubated for 1 hr at 30 C. After a washing, the plates were developed with 0.6 mg/ml of *p*-nitrophenyl phosphate substrate in 10% diethanolamine buffer (pH 9.8). Optical density values at 405 nm (OD_{405}) were taken at 1 hr with a Biotek EIA reader model EL 309 (Biotek Instruments, Burlington, VT). The threshold value for a positive reaction was an OD_{405} of greater than 0.05; this reading was at least three times the value of the healthy controls (8).

Fifteen suspected *X. albilineans* strains were assayed using the X1 MAb, an *X. albilineans* polyclonal antibody prepared by M. Irey, and MAb's of the three *X. albilineans* serovars (provided by A. M. Alvarez) to confirm identity.

TBIA. The tissue blot immunoassay procedures were obtained from M. J. Davis (Department of Plant Pathology, University of Florida, Homestead). These were modifications of those developed for detecting the ratoon stunting disease pathogen of sugarcane (7), *Clavibacter xyli* subsp. *xyli*, in which the vascular fluids containing the bacterial pathogen are collected on nitrocellulose membranes and serologically detected. A 1-cm core of tissue from each internode sampled from plants from the Sugarcane Field Station was cut in 1-cm lengths, and the cut end was placed on a nitrocellulose membrane and centrifuged at 1,700 *g* for 15 min. The membranes were then dried for 1 hr at 80 C and stored, usually for less than 10 days, until processed.

Processing was conducted on a rotary shaker at 60 rpm. The nitrocellulose membranes were blocked for 30 min in Tris buffer (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) plus blocking agent, rinsed in Tris buffer for 1 min, and incubated for 1 hr in primary antibody diluted in Tris buffer. The membranes were then rinsed three times each for 5 min in Tris buffer and incubated for 1 hr in the secondary antibody diluted in Tris buffer. After incubation in the secondary antibody, the membranes were rinsed twice and incubated in substrate solution in the dark for 20–30 min until dark blue dots appeared on the membranes where the bacteria from infected vascular bundles were deposited.

The substrate solution was prepared by adding 30 mg of naphthol AS phosphate (Sigma) in 0.5 ml of *N,N*-dimethylformamide to 100 ml of 0.2 M Tris buffer (Tris substrate buffer, pH 9.1) for staining a 100-cm^2 membrane. Immediately before use, 0.1 g of Fast Blue BB Salt and 0.5 ml of 0.1 M magnesium chloride solution were added to the 100 ml of Tris buffer. The Tris substrate buffer was used immediately, and the reaction was conducted in the dark. After incubation in the substrate solution, the membranes were rinsed in 1.05% sodium hypochlorite solution to remove a general yellowish brown discoloration, followed by a water rinse and drying between blotter paper. After processing, the membranes showed blue-stained dots where vascular bundle fluids infected with leaf scald had deposited *X. albilineans* during centrifugation. Impressions of the vascular bundles could be seen with a stereomicroscope, which aided counting the number of vascular bundles containing *X. albilineans*.

Isolation of bacteria. *X. albilineans* was isolated from a portion of the same vascular sap samples used for ELISA determinations, either by streaking or by dilution plating on Wilbrinks agar (12) amended with 5.0 g/L of yeast extract. Plates were rated on a 0–4 scale based on the amount of *X. albilineans* growth, where 0 = no colony, 1 = one or two colonies, 2 = three to 10 colonies, 3 = 11–200 colonies, 4 = >200 colonies and massive growth. *X. albilineans* was identified primarily on the basis of colony morphology and growth rate; suspect colonies were tested further by ELISA.

RESULTS

ELISA. A threshold OD_{405} of 0.05 was selected to indicate the presence of *X. albilineans*, since all samples with values greater than 0.05 were positive by isolation and/or TBIA methods. The 0.05-OD_{405} level was at least three times the level of values obtained from all the 142 asymptomatic stalk samples that were negative by isolation and TBIA techniques and also at least three times that of the healthy controls (8).

All of the 15 representative suspected *X. albilineans* strains tested with the five different antibody preparations were positive in ELISA with MAb X1, the *X. albilineans* polyclonal antiserum, and the MAb specific for *X. albilineans* serovar 1 but not those specific for serovars 2 and 3. Ten representative non-*X. albilineans* bacterial strains recovered from vascular fluids were all negative by ELISA.

Sampling. *X. albilineans* was detected more readily in samples from certain stalk positions in both asymptomatic and symptomatic stalks. Samples from position 3 of symptomatic stalks assayed 80% positive by ELISA, whereas positions 1, 2, and 4 assayed 63, 70, and 71%, respectively. The upper portion of the stalk, positions 3 and 4, gave higher OD_{405} readings, averaging 0.401 and 0.363, respectively, than positions 1 and 2, which averaged 0.276 and 0.321, respectively. Likewise, positions 3 and 4 of symptomatic stalks averaged 24.2 and 24.3 infected vascular bundles, respectively, compared with 18.6 and 20.6, respectively, for positions 1 and 2. With TBIA of symptomatic stalks, the frequency of positives was above 90% for all positions, averaging 96.8, 90.3, 93.5, and 90.3% for positions 1, 2, 3, and 4, respectively. However, isolation did not detect differences in bacterial populations from different positions on symptomatic stalks. With ELISA of asymptomatic stalks, 9.4% of the samples were positive for position 3, whereas less than 3% were positive for the other stalk positions. With TBIA of asymptomatic stalks, 20.0, 35.0, 26.3, and 18.4% of the samples were positive for *X. albilineans* for positions 1, 2, 3, and 4, respectively. Stalk position 3 was selected on the basis of overall results for further sampling to compare the assay methods. Position 3 generally gave more vascular fluids than position 4 when centrifuged.

Detection in symptomatic vs. asymptomatic stalks. When further sampling of position 3 was done, *X. albilineans* was detected much more often in symptomatic stalks than in asymptomatic stalks (Table 1). Depending on the type of assay, the frequency of detection ranged from 67.0 to 98.9% in symptomatic stalks and from 6.3 to 26.6% in asymptomatic stalks. Symptomatic stalks also gave higher assay readings than asymptomatic stalks. On the basis of dilution plating and streaking vascular sap, populations of *X. albilineans* were higher in symptomatic stalks than in asymptomatic stalks. ELISA OD_{405} values averaged 0.498 for the positive symptomatic stalks and only 0.346 for the positive asymptomatic stalks. Likewise, the number of infected vascular bundles detected by TBIA averaged 24.0 in symptomatic stalks vs. 8.5 in positive asymptomatic stalks.

Comparison of assay methods. Isolation detected *X. albilineans* in 67.0% of the symptomatic stalks, ELISA in 90.4%, and TBIA in 98.9% (Table 1). ELISA failed to detect the pathogen in nine samples from symptomatic stalks; isolation detected the pathogen in two of the nine and TBIA, in eight.

Isolation detected *X. albilineans* in 26.6% of the asymptomatic stalks, ELISA (0.05 OD threshold) in 6.3%, and TBIA in 16.2%. Of the 79 asymptomatic stalks positive for *X. albilineans*, seven were detected by all three techniques, 15 by two techniques, and 58 by only one method. Isolation had the highest frequency of detection (52 of the 79 samples).

DISCUSSION

Our results indicate that the position sampled influences the frequency of detection of *X. albilineans* in both symptomatic and asymptomatic stalks. Internodes 62.5% up from the base of the stalk were the best to sample for *X. albilineans*. Generally higher frequencies of *X. albilineans* detection and higher assay values

were obtained from this position. This position is also where abundant side shoots are found on mature stalks with symptoms. This is in contrast to the optimal sampling location for the ratoon stunting disease pathogen of sugarcane, *C. x. xyli*, which is found at highest concentrations in the basal internodes (7).

The detection of *X. albilineans* by isolation was hampered by other bacteria inhabiting the vascular fluids that often inhibited *X. albilineans* and also grew faster on Wilbrink's agar than did *X. albilineans*. In general, these bacteria were more populous in symptomatic stalks than in asymptomatic stalks. This probably accounts for the lower rate of detection by isolation than by TBIA and ELISA in symptomatic stalks and a higher detection rate by isolation than by TBIA and ELISA in asymptomatic stalks. A recently developed *X. albilineans* selective medium (6) may result in higher levels of detection and better agreement between isolation and TBIA in frequency of detection.

The detection threshold of *X. albilineans* in plant extracts from sugarcane usually has not been reported in terms of population numbers. Moffett and Croft (10) reported that the sensitivity of ELISA was comparable to that of inoculating indicator plants. Leoville and Coleno (9) were able to detect *X. albilineans* in asymptomatic stalks with immunofluorescence. In our tests, ELISA and TBIA detected *X. albilineans* at levels as low as 10^5 cfu/ml, but only about 50% of the time.

The threshold detection level in *X. albilineans* from pure cultures has been reported (3) to be 1, 10, and 10^5 cfu/ml with ELISA, latex flocculation, and immunofluorescence, respectively. These figures are significantly lower than detection limits reported for bacteria with use of similar methods. In other systems, *X. albilineans* was detected at populations of 10^3 cfu/ml with use of selected MAb's where the threshold of other antibodies was 10^5 cfu/ml (16). ELISA detected levels of *X. albilineans* from pure culture at 2×10^5 cfu per well (8), which is near the threshold determined for the ratoon stunting disease pathogen, *C. x. xyli* (11,17). ELISA has detected levels of 10^4 and 10^5 cfu/ml in other host-pathogen systems (2). In our study, TBIA appeared to be more sensitive than ELISA, since it had more positives from both symptomatic and asymptomatic stalks than ELISA. A possible reason is that bacteria in sap of infected vascular bundles is concentrated in discrete spots on the nitrocellulose membranes when TBIA is used, whereas sap from infected vascular bundles is diluted with sap from noninfected vascular bundles when ELISA is used.

The detection methods evaluated in this paper may help ascertain resistance during screening of sugarcane cultivars. TBIA is used to determine resistance levels for ratoon stunting disease of sugarcane, with the pathogen population and the number of vascular bundles containing *C. x. xyli* correlated with resistance (5,7). Whether the number of vascular bundles infected with *X. albilineans* is correlated with leaf scald resistance needs to be determined. TBIA along with ELISA and isolation detection methods could be used to help assess resistance levels by determining the levels of latently infected plants after inoculation. At present, only visual symptom expression is used to evaluate resistance, and the possibility that a high percentage of asymptomatic stalks may be infected with *X. albilineans* is ignored.

Although TBIA was 98.9% effective in detecting *X. albilineans* in symptomatic sugarcane stalks, no method was as effective in asymptomatic stalks. TBIA and isolation techniques could, however, help to eliminate or reduce the spread of the pathogen. Isolation was probably most effective in detecting *X. albilineans* in asymptomatic stalks because these stalks had lower populations of other bacteria. Therefore, TBIA and isolation methods should be used in combination in sugarcane quarantines and in determining levels of *X. albilineans* in seed cane.

Table 1. Comparison of isolation, ELISA, and TBIA techniques for detection of *Xanthomonas albilineans* in symptomatic and asymptomatic sugarcane stalks

Technique			Symptomatic stalks		Asymptomatic stalks	
Isolation	ELISA	TBIA	No.	%	No.	%
+	+	+	61	64.9	7	3.2
+	+	−	0	0	2	0.9
+	−	+	2	2.1	9	4.1
−	+	+	24	25.5	4	1.8
−	−	+	6	6.4	16	7.2
−	+	−	0	0	1	0.5
+	−	−	0	0	41	18.5
−	−	−	1	1.1	142	63.9
Total for each method						
Isolation			63	67.0	59	26.6
ELISA			85	90.4	14	6.3
TBIA			93	98.9	36	16.2
Total sampled			94		222	

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