Evaluation of Selective Media and Immunoassays for Detection of *Xanthomonas albilineans*, Causal Agent of Sugarcane Leaf Scald Disease

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ABSTRACT

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A selective medium (XAS medium) was developed for the isolation of *Xanthomonas albilineans* (Ashby) Dowson, which causes leaf scald disease of sugarcane. XAS medium supported high plating efficiencies of the pathogen. The growth rate, morphology, and pigmentation of colonies on the medium were useful differential characteristics to identify the pathogen. XAS medium consisted of a modification of Wilbrink's medium that was supplemented with 5 g of KBr, 100 mg of cycloheximide, 2 mg of benomyl, 25 mg of cephalexin, 30 mg of novobiocin, and 50 mg of kasugamycin per liter. The pathogen was isolated on XAS medium with greater than 98% frequency from symptomatic sugarcane in Florida, Guadeloupe, and the Dominican Republic, and was isolated less frequently from asymptomatic sugarcane. Plating efficiencies of 69–127% were obtained on XAS medium for 30 strains of the pathogen collected previously from locations throughout the world. Selective isolation and serological methods for detection were compared. Two methods, dilution plating of sap extracts from stalks and blotting of freshly cut surfaces of stalks, were used to inoculate XAS medium. Three enzyme immunoassays (EIA) were used, the tissue-blot EIA, stalk-blot EIA, and dot-blot EIA. The pathogen was detected consistently with each method in all 27 symptomatic stalks, with the exception of one stalk with the stalk-blot EIA. The pathogen was detected sporadically in 27 asymptomatic stalks collected from the same 27 plants. Similar detection frequencies were obtained with the dilution-plate and tissue-blot EIA methods. Detection by the other methods was approximately 7.4–14.8% less. None or low pathogen population sizes were detected in asymptomatic stalks. Contaminating bacteria interfered with selective isolation, especially when the stalk-blot inoculation method was used and when pathogen population sizes were small. Brief surface disinfestation of stalks with alcohol combined with selective isolation was found to produce satisfactory results. Selective isolation methods provided valid alternatives to immunoassays for detection of *X. albilineans*. Additionally, XAS medium enhanced plate-count procedures for estimating viable population sizes of the pathogen. The selective isolation and serological methods should enable development and implementation of more effective management practices for leaf scald disease.

Leaf scald disease of sugarcane is caused by the bacterium *Xanthomonas albilineans* (Ashby) Dowson, and it occurs in most sugarcane-producing areas of the world (15). In areas within Africa, Australia, and South America, management of leaf scald has been a continual economically important aspect of sugarcane production (12). The disease has generally been of lesser importance in areas with mild oceanic climates. Recently, however, the occurrence and incidence of leaf scald in commercial cultivars has increased at alarming rates in some areas with oceanic climates, such as Florida, Guadeloupe, Mauritius, and the Dominican Republic, causing concern that the disease will become a limiting factor in sugarcane production and variety development in these regions.

The reasons for the recent outbreaks of leaf scald have not been ascertained. Variation of the pathogen might be responsible in some situations. Strains of *X. albilineans* that apparently spread readily by aerial means, unlike previous strains, which are disseminated by mechanical means, have been encountered recently in Mauritius (2). In Florida and the Dominican Republic, two genetically distinct populations of strains have been identified based on comparison of strains by analyzing genomic DNA polymorphisms produced with rare-cutting restriction enzymes and separated by pulsed-field gel electrophoresis (4; M. J. Davis, *unpublished*). Other factors, such as unusual environmental conditions and the introduction into commercial production of more susceptible cultivars, might contribute to the increased prevalence of leaf scald in some areas.

The impact of leaf scald on sugarcane production can be minimized by appropriate management practices. Genetic resistance to the disease is heritable and is the most effective and economic means of control. Leaf scald also can be managed by production and careful selection of disease-free planting material and by proper sanitation to prevent spread, although such programs are more costly and difficult to implement and maintain. Quarantine programs exist in most production areas to prevent introduction of the pathogen or new strains of the pathogen.

Even with strict management practices, leaf scald disease remains problematic primarily from failure to detect the pathogen in asymptomatic sugarcane. Latent infections are common and hinder disease management. Isolation of the pathogen in culture has had limited application as a means of detection because of the fastidious nature of the pathogen and the lack of efficient isolation techniques. Serological techniques have been developed for the detection of *X. albilineans* (1,3,8,9,11,16) and are quite successful with symptomatic tissues. Their usefulness in detecting latent infections is questionable. Comstock and Irey (3) suggested that a combination of isolation in culture and serology might be necessary for efficient detection of the pathogen; however, their results were obtained with a nonselective isolation medium.

In general, more effective means to detect, identify, and quantify populations of *X. albilineans* are needed to facilitate the study and management of leaf scald. The objective of this study was to develop a selective medium that might help to fulfill this need. Additionally, three enzyme immunoassays were used to evaluate the efficiency of selective isolation and of serological methods in detecting the pathogen. A brief report has been published describing the stalk-blot inoculation method and selective media for isolation of *X. albilineans* (6).

MATERIALS AND METHODS

Bacterial strains. Strains of *X. albilineans* used in this study included strains CP5 and F6R from Florida and those listed in Table 1. Strains CP5 and F6R were used in the initial development of

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selective media. Both strains belong to serovar 1 as described by Rott et al (14), but they represent two genetically distinct populations of the pathogen based on restriction fragment length polymorphisms (4). Strains were maintained in culture at 28 C on a modified Wilbrink's (MW) medium with the following composition: distilled or deionized water, 1 L; Bacto Peptone, 5 g; sucrose, 10 g; $K_2HPO_4 \cdot 3H_2O$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; Na_2SO_3, 0.05 g; and Bacto agar, 15 g.

Development of selective media. The MW medium and PD3 medium (5), each supplemented with 2 mg/L of benomyl and 100 mg/L of cycloheximide to limit fungal growth, were used as basal media for the development of selective media. Antimicrobial compounds listed in the Results and Discussion section were added alone or in various combinations to the basal medium and tested for their effects on the plating efficiency of strains CP5 and F6R. One-hundred-fold serial dilutions of turbid suspensions of the strains were prepared in 0.01 M phosphate-buffered saline (PBS) (0.85%), pH 7.2; and test media were inoculated in triplicate using 0.01-ml portions of the dilutions. MW or PD3 medium with and without cycloheximide and benomyl were inoculated as controls. Colony size and number were measured after incubation for 7 days at 28 C. Individual compounds or combinations of compounds were considered inhibitory to *X. albilineans* if plating efficiency was 1% or less than that of the control plates.

In the early stages of media development, selective inhibition of a wide variety of nontarget microorganisms was crudely examined by inoculating test media with dilute soil suspensions and observing the amount and diversity of microbial growth that developed (*results not shown*). In later stages, formulations were screened by comparing relative effectiveness and selectivity of isolation of *X. albilineans* directly from infected sugarcane, using procedures described below.

The following selective media were developed: the basal selective (BS) medium composed of MW medium supplemented with 5 g/L of KBr, 100 mg/L of cycloheximide, 2 mg/L of benomyl, 25 mg/L of cephalexin, and 30 mg/L of novobiocin, and the complete *X. albilineans* selective (XAS) medium composed of the BS medium supplemented with 50 mg/L of kasugamycin. In preparation of these media, benomyl and KBr were added to the MW medium, and the pH was adjusted to 6.8 prior to autoclaving. Benomyl was added as a stock containing 0.02 g of Benlate 50DF in 5 ml of acetone to obtain 4 mg/L of Benlate (2 mg a.i.). The other ingredients were filter sterilized as 1% aqueous stock solutions and added after the autoclaved portion of the medium was allowed to cool to ca. 50 C.

Evaluation of selective media. In one experiment, the efficiency and selectivity of BS medium, XAS medium, and XAS medium supplemented with 200 mg/L of sodium deoxycholate (XAS+D medium) were directly compared to one another and to those of the MW basal medium supplemented only with 5 g/L of KBr, 100 mg/L of cycloheximide, and 2 mg/L of benomyl (MWB medium). The latter medium was used to isolate both *X. albilineans* and other bacteria without interference from fungal contaminants. Five mature sugarcane stalks, which had been harvested 16 mo earlier and stored at −40 C, were used as a source of inoculum. The stalks had been determined to be infected with *X. albilineans* prior to freezing both by isolation of the pathogen and by serological methods described herein. Internodes in the lower third of five stalks were sampled. A portion of each stalk containing an intact internode was cut from the frozen stalk. After thawing at room temperature, the stalks were cut transversely toward one end of an internode with pruning shears. A longitudinal core, 2–3 cm long, was then removed from the central portion of each internode using a sterile 10-mm-diameter cork borer. The cores were placed individually in sterile conical centrifuge tubes and centrifuged at 3,000 rpm for 10 min. Sap collected by centrifugation in this manner was diluted serially in 10-fold steps with sterile PBS, and 0.1-ml aliquots of the undiluted and diluted sap were spread in duplicate with a sterile glass rod on plates of media. Inoculated media were incubated for 7–10 days at 28 C. Colonies of *X. albilineans,* and colonies of other bacteria as a group, were counted at an appropriate dilution level to obtain estimates of population sizes. The lowest dilution level at which colonies of *X. albilineans* could readily be detected, regardless of contaminants, was recorded for each duplication of the dilution series on each medium. The experiment was repeated the following week with another portion of each stalk.

Table 1. Recovery of different strains of *Xanthomonas albilineans* on selective medium (XAS) as compared to recovery on modified Wilbrink's medium (MW)

Strain number	Geographic origin	Source[x]	Recovery[y] (%)	Colony diameter[z] (mm)	
				XAS	MW
AUS 23A	Australia	1 (UQM 869)	85	3.0	2.5
BRB 34A	Barbados	2	91	1.0	1.0
BRA 49A	Brazil	3	86	2.0	2.1
HVO 05A	Burkina Faso	2	106	2.4	2.1
CMR 11A	Cameroon	2	90	2.7	2.5
FJI 79A	Fiji	4 (NCPPB 2969)	69	2.0	1.8
USA 19A	Florida	5	100	1.3	1.2
USA 48A	Florida	6	113	2.0	2.0
GLP 07A	Guadeloupe	2	85	2.4	2.2
GLP 55A	Guadeloupe	2	127	1.0	1.0
GUY 69A	Guyana	4 (NCPPB 1050)	93	2.1	2.2
HAW 73A	Hawaii	4 (NCPPB 1830)	74	2.3	2.1
IND 17A	India	7	93	1.9	2.0
CIV 42A	Ivory Coast	2	104	2.0	2.2
JPN 88A	Japan	2	91	2.0	2.0
KEN 06A	Kenya	2	96	1.6	1.1
MDG 65A	Madagascar	4 (NCPPB 896)	95	1.8	1.7
MWI 74B	Malawi	8 (ICMP 8679)	98	1.1	1.0
MTQ 58A	Martinique	2	75	2.0	2.0
MTQ 78A	Martinique	4 (NCPPB 2503)	104	1.7	1.7
MUS 14A	Mauritius	2	87	1.7	1.8
REU 08A	Reunion	9 (CFBP 1980)	94	2.9	2.9
ZAF 28A	South Africa	10	92	1.9	2.0
LKA 70B	Sri Lanka	8 (ICMP 8677)	87	1.0	1.0
KNA 03A	St. Kitts	9 (CFBP 1213)	107	2.1	2.0
KNA 91A	St. Kitts	2	92	2.0	1.9
LCA 63A	St. Lucia	4 (NCPPB 526)	92	2..1	2.2
TWN 52A	Taiwan	11	127	1.0	1.0
ZAR 47A	Zaire	2	88	1.6	2.0
ZIM 71A	Zimbabwe	4 (NCPPB 1737)	95	1.2	1.6
Mean			95	1.9	1.8

[x] 1 = A. C. Hayward, University of Queensland, Australia; 2 = Plant Pathology Laboratory, CIRAD-CA, Montpellier, France; 3 = R. A. Sordi, Brazil; 4 = D. Stead, National Collection of Plant Pathogenic Bacteria, Harpenden, England; 5 = J. L. Dean, Florida; 6 = M. J. Davis, Florida; 7 = K. K. Prasada Rao, India; 8 = J. M. Young, International Collection of Microorganisms from Plants, Aukland, New Zealand; 9 = L. Gardan, Collection Francaise de Bactéries Phytopathogènes, Angers, France; 10 = R. Bailey, South Africa; 11 = C. T. Chen, Taiwan. Each strain number in parentheses corresponds to an official registered strain number.

[y] Each value is the average of three replicates and represents the number of colonies obtained on XAS medium as a percentage of those obtained on MW medium.

[z] Each value is the average of 10 colonies.

The efficiency of recovery of a wide variety of strains listed in Table 1 on the XAS medium was compared with recovery on MW medium. Suspensions of each strain in sterile water were adjusted to 0.5 optical density at 530 nm. Plates of each medium were inoculated by spreading 0.05 ml of 10-fold serial dilutions of the suspensions with a sterile glass rod. Three plates of each medium were inoculated with each dilution. Colonies were counted, and the diameter of 10 random, widely spaced colonies for each treatment was measured after a 7-day incubation at 28 C.

Immunoassay sample preparation. Sample preparation for tissue-blot enzyme immunoassay (TB-EIA) was conducted as described by Harrison and Davis (7) with modification. Briefly, two 10-mm-thick cross sections were excised from the 10-mm-diameter stalk cores using a device containing parallel-mounted, thin metal blades. Sections were carefully placed within wells of a filter-holding apparatus onto the surface of a nitrocellulose membrane filter. The filter apparatus consisted of two 13 × 8.7 × 1 cm plastic plates. The top plate had 30, 11-mm holes in a five by six array which formed wells when the two plates were bolted together. A single membrane filter was stacked on top of two sheets of absorbent paper within the apparatus. Each filter apparatus was designed to have the same length and width as a standard 96-well microtiter tray. Using four rotor attachments, each of which was designed to carry a microtiter tray, the apparatus loaded with tissue sections were centrifuged at 3,000 rpm for 10 min. After centrifugation, tissue sections were discarded. The membrane filters were removed from the filter apparatus, air dried, and incubated at 80 C for 1 hr to kill and fix cells of the pathogen deposited on the surface. Filters were subsequently subjected to the indirect EIA described below. Impressions of the tissue sections were made in the membrane filter during centrifugation, enabling visualization of individual vascular bundles and the periphery of each section. Blue-stained impressions of vascular bundles indicated the presence of *X. albilineans*.

Sample preparation for stalk-blot enzyme immunoassay (SB-EIA) was performed using membrane filters that had been inoculated by the stalk-blot procedure, placed on the surface of culture media, and incubated. Protocols for membrane preparation prior to the indirect EIA were adapted from those used for immunological colony screening (17). Before inoculation, 80 × 80 mm nitrocellulose filters were moistened with deionized water, placed between Whatman 3MM filter paper, wrapped in aluminum foil, sterilized by autoclaving, and put while damp on the surface of media in 90 × 90 mm plastic culture dishes. After inoculation and incubation, membranes were removed from the culture media, soaked twice for 15 min each time at room temperature in TNT buffer (10 mM Tris, 150 mM NaCl, and 0.05% Tween 20, pH 8.0), washed twice with agitation (68 rpm) for 30 min each time at room temperature in TNT buffer, and subjected to the indirect EIA described below. Blue-stained areas where bacterial colonies had been indicated the presence of *X. albilineans*.

Sample preparation for dot-blot enzyme immunoassay (DB-EIA) was performed using the first 10-fold dilution of the sap extracts that had been prepared for dilution plate isolations. Diluted extract (0.1 ml) was applied per well of a dot-blot vacuum manifold containing a nitrocellulose membrane filter. After sample application and filtration, the membranes were subjected to the indirect EIA described below.

Serological assays. Whole rabbit antisera prepared against strain CP5 of *X. albilineans* from Florida were used in all serological assays. Strain CP5 belongs to serovar 1 as described by Rott et al (14). Two other serovars of *X. albilineans* have been identified in other geographic locations, but only serovar 1 has been found in Florida (P. Rott, M. J. Davis, and P. Baudin, *unpublished*). The antiserum had a titer of at least 1:1,024 in agglutination tests with strain CP5 and titers less than 1:64 in similar tests with strains HV5 and G7 of *X. albilineans*, which belong to serovars 2 and 3, respectively.

The same indirect method of EIA was used in TB-, SB-, and DB-EIA. Membranes were first blocked in TBS-BSA-Tween (50 mM Tris, 150 mM NaCl, 1% bovine serum albumin fraction 5, and 0.05% Tween 20, pH 7.5) for 90 min with agitation (50 rpm) at room temperature. Next, the membranes were incubated at room temperature for another 90 min without agitation in *X. albilineans* antiserum diluted in TBS-BSA-Tween to 1:2,000. Membranes were rinsed briefly three times in the buffer without BSA (TBS-Tween) and then soaked three times in TBS-Tween for 10 min each. Membranes were then incubated for 90 min at room temperature without agitation in goat anti-rabbit IgG labeled with alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, IN) and diluted 1:6,000 in TBS-BSA-Tween. Membranes were rinsed briefly and then soaked twice for 10 min each in TBS-Tween. The membranes were soaked in substrate buffer for 10 min and then subjected to the fast blue/naphthol phosphate substrate as described (7).

An agglutination assay was used routinely to check the identity of suspected isolates of *X. albilineans*. Moderately turbid bacterial suspensions in PBS were mixed in the wells of microtiter dishes with an equal, 100-µl volume of antiserum diluted 1:100 with PBS. The mixture was incubated at room temperature for 15–60 min and observed for agglutination of bacteria using a stereo microscope.

Comparison of isolation and serological methods. Twenty-seven symptomatic plants of cultivar CP 78-1247 were sampled in a commercial field in Florida. Approximately 8-mo-old stalks, one with foliar symptoms of leaf scald and one without symptoms, were taken from each plant. Internodes in the lower third of each stalk were cut transversely with sterile pruning shears. Freshly cut surfaces of each stalk were blotted onto MW medium, XAS medium, and the surface of sterile nitrocellulose membrane filters (BA85, Schleicher and Schuell, Keene, NH) placed on the surface of these two media. A longitudinal core was then removed from the central portion of one of the internodes from each stalk using a sterile 10-mm-diameter cork borer. A portion of each core was used for TB-EIA. Another portion, 2–3 cm long, was placed in a sterile conical centrifuge tube and centrifuged at 3,000 rpm for 10 min. Sap collected by centrifugation in this manner was used both in DB-EIA and in isolation attempts on the MW and XAS media. In isolation attempts, the sap was diluted serially in 10-fold steps with PBS, and the media were inoculated by spreading 100-µl portions of each dilution over the surface of the media with a sterile glass rod. Inoculated media were incubated for 7–10 days at 28 C.

RESULTS AND DISCUSSION

Two selective media have been reported for isolation of *X. albilineans* from sugarcane prior to this study (10,13). In our hands, however, Persley's medium (10) lacked selectivity, and the medium of Rivera et al (13) did not support the growth of strains CP5 or F6R. When individual selective ingredients of the medium of Rivera et al were analyzed at the recommended concentrations, we found that brilliant green dye was toxic to strains CP5 and F6R, and oxacillin inhibited strain F6R but not strain CP5.

A number of compounds were tested for possible incorporation into selective media for *X. albilineans*. Compounds that were not inhibitory to either strain CP5 or F6R included benomyl (2 mg/L), bile salts (80 mg/L), cephalexin (50 mg/L), crystal violet (1 mg/L), cycloheximide (100 mg/L), deoxycholate (100 mg/L), kasugamycin (50 mg/L), KBr (20 g/L), methyl green (10 mg/L), novobiocin (30 mg/L), tobramycin (0.4 mg/L), and vancomycin (12.5 mg/L). Compounds that were inhibitory to both strains CP5 and F6R included brilliant green (3.4 mg/L), colistin sulfate (10 mg/L), eosin (2 g/L), fluorouracil (3 mg/L), gentamycin (2.5 mg/L), KIO_3 (100 mg/L), methylene blue (400 mg/L), neomycin (10 mg/L), trimethoprim (15

mg/L), and triphenyl tetrazolium chloride (50 mg/L). Compounds that were inhibitory to strain F6R, but not strain CP5, included ampicillin (0.5 and 25 mg/L), bacitracin (12.5, 25, 50, and 100 mg/L), deoxycholate (200 mg/L), methicillin (10 mg/L), oxacillin (30 mg/L), and vancomycin (25 mg/L).

Testing of various combinations of potentially useful antibiotics resulted in the formulation of the BS and XAS media. Benomyl and cycloheximide were found to inhibit most fungal growth on the MW medium, and addition of novobiocin and cephalexin substantially reduced the incidence of bacterial contaminants. Potassium bromide was incorporated because it slightly stimulated faster growth and pigmentation of colonies, thus facilitating the use of the pale yellow pigmentation of *X. albilineans* as a differential characteristic. This medium, designated the basal selective (BS) medium, supported a higher plating efficiency for *X. albilineans* while significantly reducing the number of nontarget bacteria compared with the same medium without cephalexin and novobiocin (MWB medium, Table 2). The addition of kasugamycin to BS medium (XAS medium) tended to further enhance selectivity without loss of plating efficiency (Table 2). Deoxycholate was initially deemed useful because it inhibited some nontarget bacteria that spread rapidly over the surface of media in some tests; however, deoxycholate was toxic to strain F6R at 200 mg/L but not at 100 mg/L. Furthermore, deoxycholate at 200 mg/L in the XAS medium failed to enhance selectivity and significantly reduced the plating efficiency for *X. albilineans* upon isolation from sugarcane (Table 2). In other tests, addition to the BS or XAS media of the other potentially useful compounds listed above that did not inhibit either test strain of *X. albilineans* did not substantially improve the selective or differential characteristics of the medium.

XAS medium should be useful for isolating *X. albilineans* in different geographic locations around the world. Thirty strains of the pathogen from various geographic origins grew well on XAS medium (Table 1). Furthermore, XAS medium and the stalk-blot method of inoculation were used successfully to detect the pathogen in sugarcane in Guadeloupe and the Dominican Republic, as well as in Florida. In Guadeloupe, 120 stalks of the susceptible clone B 69-566 were sampled at random from a commercial field during May 1991. The pathogen was isolated from 15 of 16 stalks with typical side-shoot symptoms of leaf scald and from five symptomless stalks. The pathogen was isolated also from three of 253 symptomless stalks from sugarcane nurseries. In the Dominican Republic, eight sugarcane clones grown in commercial fields or breeding plots were surveyed in June 1991. The pathogen was isolated from all 36 symptomatic stalks and six of 32 asymptomatic stalks sampled from 57 stools in 10 different fields. In the six cases where the pathogen was isolated from asymptomatic stalks, four of the six stools sampled had no stalks with any visual symptoms.

XAS medium proved useful when different isolation and serological techniques were compared directly for detection of *X. albilineans* in symptomatic and asymptomatic sugarcane stalks from a commercial planting of cultivar CP 78-1247 in Florida. Growth of bacterial contaminants was observed on XAS medium near the periphery of the stalk imprints and at lower dilutions on spread plates; however, this growth was limited and did not interfere with the detection of *X. albilineans* to any noticeable extent. In contrast, bacterial and fungal contamination was often an overwhelming problem when isolations were made with the MW medium. Due to the MW medium being overrun by contaminants, only 3.7% (2/54) and 85.2% (46/54) of the isolation attempts could be scored for the presence or absence of *X. albilineans* using the stalk-blot and dilution-plate methods of isolation, respectively. Contaminants were eliminated frequently by dilution of sap extracts, permitting isolation of *X. albilineans* without the use of selective medium, especially when large populations of the pathogen were present. The agreement between results obtained with dilution plating on XAS and MW media was 93.5% (43/46) for detection of the pathogen, and there was a 99.7% correlation between population measurements obtained with the two media.

In most cases, isolates of *X. albilineans* were identified in culture without difficulty on the basis of morphology, size, and pigmentation of colonies. In some cases, identification on the basis of colony characteristics was facilitated by the transfer of isolates to fresh medium. Variation in the size of *X. albilineans* colonies, as previously described (12), was observed frequently. Two colony sizes were evident, especially among widely spaced colonies on dilution or streak plates. Serological agglutination tests were consistently positive for isolates identified as *X. albilineans* on the basis of colony characteristics; whereas the tests were consistently negative for contaminants.

Selective isolation of the pathogen was more efficient with the dilution-plate method than with the stalk-blot method. The pathogen was detected in 66.7% (36/54) of the samples by the dilution-plate method and in 51.9% (28/54) of the samples by the stalk-blot method. Interference from surface contaminants may have accounted for the lower efficiency of the stalk-blot method of inoculation. Based on colony morphology and color, two unidentified bacteria, one having white colonies and the other having yellow colonies, were the most frequently isolated contaminants in samples from Florida, Guadeloupe, and the Dominican Republic. The intensity of the yellow-colored pigmentation of the one bacterial contaminant was greater than the pale yellow pigmentation of *X. albilineans*. Simple cleaning of stalk surfaces with an ethanol-soaked cloth greatly reduced contamination and thus facilitated pathogen detection (*data not shown*).

For the most part, *X. albilineans* was detected easily in symptomatic sugarcane stalks regardless of the serological or isolation methods used (Table 3); however, detection of the pathogen from asymptomatic stalks varied. The variability was most likely due to low population sizes of the pathogen in asymptomatic stalks. The mean pathogen population in sap extracts from symptomatic stalks was 2.8×10^{10} cells per milliliter, whereas the population was only 1.1×10^5 cells per milliliter from asymptomatic stalks. Similarly, the mean total number of colonized vascular bundles determined by TB-EIA of two tissue sections

Table 2. Efficiency and selectivity of modified Wilbrink's medium (MW) supplemented with potassium bromide and different combinations of selective ingredients for isolation of *Xanthomonas albilineans* from sugarcane[w]

Medium[x]	Detectable population size[y] (Log_{10} cfu/ml)		Minimum dilution permitting detection (log_{10} dilution)[z]
	X. albilineans	Other bacteria	
MWB	9.1 a[y]	7.9 a[y]	2.4 a[y]
BS	9.1 a	3.7 b	0.7 bc
XAS	9.2 a	3.3 b	0.0 c
XAS+D	8.8 b	3.3 b	0.9 b

[w] Media were inoculated with dilutions of sap extracts from infected sugarcane stalks to obtain estimates of the population size of *X. albilineans*, the total number of bacterial contaminants, and the minimum dilution level at which *X. albilineans* was readily detected.

[x] Abbreviations: MW basal (MWB) medium = MW with KBr (5 g/L), cycloheximide (100 mg/L), and benomyl (2 mg/L); basal selective (BS) medium = MWB with cephalexin (25 mg/L) and novobiocin (30 mg/L); complete *X. albilineans* selective (XAS) medium = BS with kasugamycin (50 mg/L); D = deoxycholate (200 mg/L).

[y] Each value represents the mean of a total of 20 determinations derived in duplicate from two internodes sampled at two different times from five infected stalks.

[z] Means in the same column followed by the same letter were not significantly different as determined by the Waller-Duncan *k*-ratio *t* test: *k*-ratio = 100.

Table 3. Direct comparison of selective isolation on XAS medium and serological methods for detection of *Xanthomonas albilineans* in sugarcane stalks. One symptomatic and one asymptomatic stalk were sampled from each of 27 plants of cultivar CP 78-1247 in a commercial planting in Florida

Observed combination of results[y]						
Leaf scald symptoms	Dilution-plate isolation	Stalk-blot isolation	Stalk-blot EIA	Tissue-blot EIA	Dot-blot EIA	Observed frequency[z]
+	+	+	+	+	+	26 (48.1)
+	+	+	−	+	+	1 (1.9)
−	+	+	−	+	+	1 (1.9)
−	+	−	+	−	−	3 (5.6)
−	+	−	−	+	+	1 (1.9)
−	+	−	−	+	−	2 (3.7)
−	+	−	−	−	+	1 (1.9)
−	+	−	−	−	−	1 (1.9)
−	−	−	+	+	−	1 (1.9)
−	−	−	+	−	−	2 (3.7)
−	−	−	−	+	−	4 (7.4)
−	−	−	−	−	+	2 (3.7)
−	−	−	−	−	−	9 (16.7)

[y] Abbreviations: EIA = enzyme immunoassay; + = positive detection; − = negative detection.
[z] Number of stalks with observed combination of results for all assay methods. In parentheses, percentage of all stalks.

from each stalk was 59.7 for symptomatic stalks and 3.7 for asymptomatic stalks. Results from TB-EIA correlated highly with results from the dilution-plate assay ($R = 0.80$, $P < 0.0001$).

The leaf scald pathogen was detected in 66.7% (36/54) of the stalks by TB-EIA. Disagreement between results of TB-EIA and the dilution-plate assay was 18.5% (10/54) and was due equally to positive and negative results. Positive results (7.4%) were obtained by TB-EIA which were not confirmed by other serological assays or by isolation on XAS medium. In two of these cases, the pathogen was isolated on MW medium but at low population levels ($<10^5$ cfu/ml). TB-EIA results for duplicate tissue sections taken from each stalk differed on the presence or absence of the pathogen in five cases. Thus, a slightly lower level of detection, approximately 62%, would have been obtained if only one section instead of two sections from each stalk had been analyzed. These discrepancies again were apparently due to sporadic detection at low colonization levels of the pathogen. A high overall correlation ($R = 0.92$, $P < 0.0001$), however, existed between the number of colonized vascular bundles in duplicate sections of stalks.

The pathogen was detected in 59.3% (32/54) of the stalks by SB-EIA and DB-EIA. Results for both of these techniques included two possible false positives. A diffuse, light blue stain was often present on membrane filters and spread out from locations of sample application. Presumably, highly antigenic, soluble antigens produced by the pathogen were stained. The stain sometimes ran over into other sample blots, making it difficult to score results. In other studies, several methods of preparing antigens for antiserum production in rabbits and different EIA protocols, including different methods of blocking nonspecific binding sites on membranes, were tested. All failed to substantially reduce the diffuse staining (M. J. Davis, *unpublished*). The presence of highly soluble antigens might be advantageous in other serological assays such as ELISA (3), but may be a confounding factor in TB-EIA and SB-EIA.

Selective isolation of *X. albilineans* using XAS medium was found to be a relatively simple and effective means of detection. Selective isolation and serology used together in various ways may improve the diagnosis of leaf scald. For example, we routinely used a relatively simple serological agglutination assay to confirm the identity of *X. albilineans* colonies isolated on XAS medium. The ease of the stalk-blot inoculation method and the specificities of selective isolation and serological detection were combined in SB-EIA. In so doing, selective isolation enriched for the presence of the pathogen and enhanced serological detection. The relative merits of the isolation and serological assays used in this study will vary depending on different applications. Altogether, the assays offer a versatile choice of options that should enable development and implementation of more effective management practices for leaf scald disease.

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