SELECTIVE ISOLATION OF XANTHOMONAS ALBILINEANS, CAUSAL AGENT OF LEAF SCALD DISEASE

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ABSTRACT
Selective media were developed for isolation of Xanthomonas albilineans, which causes leaf scald disease of sugarcane. The basal selective medium consisted of a modification of Wilbrink's medium that was supplemented with 5 g/l of KBr, 100 mg/l of cycloheximide, 2 mg/l of benomyl, 25 mg/l of cephalxin and 30 mg/l of novobiocin. For greater selectivity, this medium was further supplemented with 50 mg/l of kasugamycin and 200 mg/l of sodium deoxycholate. Detection of 200 mg/l of X. albilineans in a total of 1,988 stalks; 100 asymptomatic and 98 symptomatic; from 11 sugarcane clones was tested. To compare methods of isolation, plating dilutions of sap extracts from stalk internodes onto the basal selective medium was compared with blotting freshly cut surface of the same stalk internodes onto the complete selective medium. X. albilineans was isolated from 123 stalks, and there was 91% agreement between the results of the two isolation methods. Sections of the same stalk internodes were also examined using a tissue-blot immunoassay (TBIA). There was 87% agreement between the combined isolation results and TBIA results; 92% of the disagreement being due to false negative results in the TBIA. Subsequently, the stalk-blot isolation method has been used successfully to screen sugarcane for the presence of X. albilineans in Florida, Guadeloupe and the Dominican Republic.

Key words: Diagnosis, detection, selective media.

INTRODUCTION
Leaf scald disease of sugarcane is caused by the bacterial pathogen, Xanthomonas albilineans (Ashby) Dowson, and occurs in most sugarcane producing areas of the world (Rott et al.¹⁰). In areas within Africa, Australia and South America having more stressful continental climates, management of leaf scald has continually been and economically important aspect of sugarcane production (Ricaud and Ryan⁸). Conversely, the disease has generally been less of a problem in areas with milder
M.J. DAVIS, P.P. ROTT AND J.L. DEAN

oceanic climates. Recently, however, the occurrence and incidence of leaf scald in commercial varieties have increased at alarming rates in some areas with oceanic climates, such as Florida, Guadeloupe, Mauritius and the Dominican Republic, and there is considerable concern within the industry that the disease will become a limiting factor in sugarcane production and variety development in these regions.

The reasons for the outbreaks of leaf scald at the present time are unknown. New strains of the pathogen have been identified in Mauritius which are apparently spread by aerial means and might be responsible for recent epidemics in that country (Autrey et al.1). Whether or not such strains exist elsewhere and the importance of aerial spread of X. albilineans has not been determined. Other factors, such as environmental conditions and the introduction into commercial production of susceptible varieties, might also be creating conditions that are more conducive to the development and spread of leaf scald.

Leaf scald is a manageable disease, and its impact on sugarcane production can be minimized. Genetic resistance to the disease is highly heritable and is a most effective and economical means of control. However, more efficient methods are needed to identify and screen for resistance in commercial varieties and breeding lines, including the selection of strains of the pathogen used in the process, especially if different races or other variants of the pathogen exist and need to be considered. Leaf scald can also be managed through programs involving the production and/or careful selection of disease-free planting material and proper sanitation to prevent spread, although such programs are costly and difficult to implement and maintain.

Inherent in the study and management of leaf scald is the need to detect and identify the pathogen. The frequent occurrence of latent infections has greatly limited the usefulness of diagnosis based on visual symptoms. Furthermore, the fastidious nature of X. albilineans has limited the use of isolation on culture medium as a means of diagnosis. To overcome these problems, serological methods have been developed for the diagnosis of leaf scald (Autrey et al.1, Leoville and Coleno2, Oliveira et al.3, Ricaud et al.4, Rott et al.5). However, the need to isolate the pathogen in culture often exists. To aid in isolation, selective media have been developed for X. albilineans (Persley6, Rivera7, et al.8), but none have been widely used.

We report herein new selective media for isolation of X. albilineans from sugarcane, and the results of a field test to examine the efficiency of these media and the isolation methods used to employ them for detection of the pathogen. For ease of detection, simply blotting the freshly-cut surface of stalks onto selective medium was tested by comparison with isolation of X. albilineans on selective medium from serially-diluted stalk extracts and with serological detection using a tissue-blot immunoassay (TBIA).
MATERIALS AND METHODS

Selective media

Modified Wilbrink's medium had 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; \( NaSO_4 \), 0.05 g; Bacto-Agar, 15 g. The basal selective medium was composed of modified Wilbrink's medium supplemented with 5 g of KBr, 100 mg of cycloheximide, 2 mg of benomyl, 25 mg of cephalaxin and 30 mg of novobiocin. For increased selectivity, this medium was further supplemented with 50 mg of kasugamycin and sometimes 200 mg of sodium deoxycholate. Benomyl and KBr were added to the modified Wilbrink's medium and the pH adjusted to 6.8 prior to autoclaving. Benomyl was added as a stock containing 0.02 g Benlate 50 DF in 5 ml acetone to obtain 4 mg/l Benlate (2 mg a.i.). The other selective ingredients were filter sterilized as one percent aqueous stock solutions and added after the autoclaved medium was allowed to cool to ca. 50°C.

Plant material

To test the efficiency of diagnostic techniques, 11 breeding selections at the Sugarcane Field Station at Canal Point, Florida, USA were sampled in February 1991. Approximately equal numbers of mature stalks with leaf scald symptoms and without symptoms were chosen at random from each clone to make up 98 symptomatic stalks and 100 non-symptomatic stalks. An internode in the lower third of each stalk was sampled.

Isolation

Internodes were cut transversely toward one end with a knife or pruning shears. One freshly-cut surface of each internode was blotted onto the selective medium supplemented with kasugamycin and sodium deoxycholate. A longitudinal core was then removed from the central portion of the internode using a 10 mm diameter cork borer. One portion of each core was used in the serological assay described below. Another portion, 2 to 3 cm in length, was placed in a conical centrifuge tube and centrifuged at 3,000 rpm for 10 min. Sap collected by centrifugation in this manner was then diluted serially in one hundred fold steps with 0.01 M phosphate buffered saline (0.85%), pH 7.2. Three 0.01 ml aliquots of each sap dilution were put on the basal selective medium. Inoculated medium was incubated for 7 to 10 days at 28°C.

Serological assay

The tissue-blot enzyme immunoassay (TBA) was conducted as described by Harrison and Davis, except for modifications described below. Briefly, a 10 mm
thick cross section was excised from cores using a device containing parallel-
mounted, thin metal blades. Sections were carefully placed onto the surface of
nitrocellulose membrane filter (BA85, Schleicher and Schuell, Keene, New Hamp-
shire; USA) within wells of a filter-holding apparatus. The filter apparatus consisted
of two, 13 x 8.7 x 1 cm plastic plates. The top plate had thirty 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 (3MM,
Whatman International Ltd.; Maidstone, England) within the apparatus.

Each filter apparatus was designed to have the same length and width of a standard
96 well microtiter plate. Using four rotor attachments (no. 5782, International
Equipment Co., Needham Heights, Massachusetts, USA) each of which was
designed to carry a microtiter plate, a maximum of four devices, containing up to a
total of 120 sections, were processed at 3,000 rpm for 10 minutes in a centrifuge
(Centra-7, IEC). Since X. albilineans inhabits the xylem tissue within vascular
bundles, centrifugation caused cells of the pathogen to be deposited on the surface
of the membrane filter in discrete areas below colonized vascular bundles, while at
the same time any sap released from the plant tissue passed through the membrane
and was absorbed by the underlying paper. After centrifugation, tissue sections were
discarded, and membrane filters were removed from filter devices, air dried, and
incubated at 80°C for one hour to kill cells of the pathogen and to fix them to filters.
Filters prepared in this manner were stored up to one month at room temperature
before further serological processing.

An indirect enzyme immunoassay was used to stain cells of the pathogen deposited
onto filters. The immunoassay was conducted essentially as described by Harrison
and Davis except that, instead of purified immunoglobulin G, whole antiserum
prepared against a cell wall fraction of X. albilineans was used at a dilution of 1:1,000
as the first antibody, and volumes of reagents were increased proportionately to
accommodate the larger membrane filters. Impressions of the tissue sections were
made in the membrane filter during centrifugation, allowing individual vascular
bundles and the periphery of each section to be subsequently observed. Blue-stained
impressions of vascular bundles indicated the presence of X. albilineans.

RESULTS AND DISCUSSION

Various modifications of Wilbrink's medium are commonly used to isolate and
grow X. albilineans in culture (Ricaud and Ryan) and with the addition of antimicro-
bial compounds, have served as the basal media for selective isolation of the
pathogen from sugarcane (Persley, Rivera et al.). In addition to antifungal com-
ounds such as cycloheximide and benomyl, different antibacterial compounds
have been incorporated into the media. Persley used penicillin G to limit bacterial
contaminants; whereas, Rivera et al. used a combination of bile salts, brilliant green

479
dye and oxacillin for the same purpose. In our preliminary experiments (data not shown), the use of penicillin G alone to control bacterial contamination was inadequate, and brilliant green dye at the recommended concentration was toxic to X. albilineans. Furthermore, penicillin G, oxacillin and other penicillin-type antibiotics (methicillin and ampicillin) were inhibitory to a few strains of X. albilineans. To overcome these problems, numerous antibacterial compounds were tested at various concentrations, alone or in combination with other antibacterial compounds. The selective media reported herein contain combinations of selective ingredients which we have found useful upon further field tests.

Breeding selections at Canal Point, Florida, were sampled in the latter part of the growing season after their leaves had suffered severe cold damage, and consequently, few foliar symptoms of leaf scald remained, except for the abnormal production of side shoots. Nevertheless, X. albilineans was isolated from 98% of the symptomatic stalks. Furthermore, the pathogen was isolated from 27% of the stalks without visible symptoms. When the concentration of X. albilineans was high in the inoculum, lawns of the bacterium became visible on both selective media after three to four days of incubation; whereas, 7 to 10 days elapsed before individual colonies were detectable when concentrations were low.

Results of isolation by the dilution-plate or stalk-blot inoculation methods and of the TBIA agreed totally with each other in 85.9% of the cases (Table 1). The results of the two isolation methods agreed in 90.9% of the cases; neither method produced substantially more positive or negative results. Isolation results, disregarding the method of isolation, agreed with TBIA results in 86.9% of the cases; 92.3% of the disagreement being attributable to false-negative results of TBIA.

The basal selective medium was adequate in most situations for isolation using the dilution-plate method. Few contaminating microorganisms were detected at the dilution levels (minimum level of detection was 1 x 10⁴ colony-forming unit per milliliter of sap) used in the study. However, contamination was a problem on this medium in preliminary experiments (data not shown) when undiluted sap extracts were streaked directly on this medium with an inoculation loop. Addition of kasugamycin and sodium deoxycholate to the basal selective medium imparted an additional degree of selectivity such that the stalk-blot inoculation method was found effective. Some contamination was frequently observed around the periphery of the stalk imprint on the medium but was not a major limitation. Two contaminating bacteria, one with white colonies and the other with yellow colonies, were the most frequent contaminants. The color of the yellow bacterial contaminant was brighter than the pale yellow color of X. albilineans. It was found subsequently that by simply cleaning the surface of the stalk with an ethanol soaked cloth greatly reduced contamination.
M.J. DAVIS, P.P. ROTT AND J.L. DEAN

TABLE 1. Frequency of all possible combinations in a comparison of isolation on selective medium, using two different inoculation methods, with a serological assay for detection of *X. albilineans* in sugarcane stalks.

<table>
<thead>
<tr>
<th>Stalk-Blot Isolation</th>
<th>Dilution-Plate Isolation</th>
<th>Tissue-Blot Enzyme Immunoassay</th>
<th>No. samples</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>97</td>
<td>49.0</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>73</td>
<td>36.9</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
<td>8</td>
<td>4.0</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
<td>9</td>
<td>4.5</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>+</td>
<td>7</td>
<td>3.5</td>
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<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>1.0</td>
</tr>
</tbody>
</table>

+ = positive detection; − = negative detection.

In Guadeloupe, the stalk-blot isolation method and the basal selective medium supplemented with 50 mg/l of kasugamycin were used to examine 120 stalks that were randomly sampled in May 1991 in a commercial field grown with a susceptible clone (B 69 566). The pathogen was isolated in 15 out of 16 stalks with typical leaf scald side-shooting. It was also found in five stalks without any symptom of the disease. In the Dominican Republic, eight sugarcane clones in commercial fields and breeding plots were surveyed in June 1991 for the presence of *X. albilineans* using the same technique. The pathogen was isolated from all 36 symptomatic stalks and 6 of 32 asymptomatic stalks sampled from 57 stools in ten different fields. Where the pathogen was isolated from asymptomatic stalks, four of the six stools sampled had no stalks with symptoms.

CONCLUSION

Although different methods exist for detection of *X. albilineans* in sugarcane, the stalk-blot method of isolation on selective medium was found to be very advantageous because it is not time-consuming and therefore, allows the rapid examination of numerous samples. This is very useful in varietal screening programs or commercial fields. Furthermore, the stalk-blot method does not require sophisticated equipment and is relatively inexpensive when compared with serological methods. The method has also been successfully used by other workers in Florida,
Guadeloupe and the Dominican Republic. The isolation method and selective media
developed in this study should greatly facilitate future efforts to study and control
leaf scald disease in sugarcane.

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ISOLEMENT SELECTIF DE XANTHOMONAS ALBILNEANS
AGENT RESPONSABLE DE LA MALADIE
DE L'ECHAUDURE DE LA CANNE À SUCRE

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RESUME

Des milieux sélectifs ont été développés pour l'isolement de Xanthomonas albilineans qui cause la maladie de l'èchaudure de la canne à sucre. Le milieu de base sélectif consistait en une modification du milieu de Wibrink avec addition de 5 g/l de KBr, 100 mg/l de cycloheximide, 2 mg/l de bénomyl, 25 mg/l de céphaloxine et 30 mg de novobiocine. Pour une plus grande sélectivité, ce milieu a été complété avec 50 mg/l de Kasugamycine et 200 mg/l de déoxycholate de sodium. La détection de X. albilineans dans un total de 198 tiges, dont 100 exemptes de symptômes provenant de 11 clôtures de canne à sucre a été évaluée. Pour comparer les méthodes d'isolement, les dilutions d'extraits de sève provenant d'entrenoeuds de tiges étalées sur un milieu de base sélectif ont été comparées avec l'impression de sections de cannes fraîchement coupées au niveau des entrenoeuds des mêmes tiges sur milieu sélectif complet. X. albilineans a été isolé de 163 tiges et 91% de résultats concordants ont été obtenus par les deux techniques d'isolement. Des sections des mêmes entrenoeuds de tige ont également été examinées par la technique d'immuno analyse par impression de tissus (TBIA), 87% de résultats concordants ont été obtenus entre la méthode d'isolement et la technique TBIA; 92% des résultats non concordants étaient dus à des résultats apparemment négatifs du TBIA. Subséquemment la méthode d'isolement par impression de tiges a été utilisée avec succès pour le dépistage de X. albilineans dans la canne à sucre en Floride, Guadeloupe et République Dominicaine.

Mots clés: Diagnostic, detection, milieu sélectif.
AISLAMIENTO SELECTIVO DE XANTHOMONAS ALBILINEANS, AGENTE CAUSAL DE LA ESCALDADURA DE LA HOJA

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RESUMEN

Se desarrollaron medios selectivos para aislar la bacteria Xanthomonas albilineans que causa la enfermedad de la escaladura de la hoja. El medio selectivo básico fue una modificación del medio de Wilbrinks, suplementado con 5 g/l de KBr, 100 mg/l de cycloheximide, 2 mg/l de benomyl, 25 mg/l de cephalixin y 30 mg/l de novobiocon. Para aumentar su selectividad, este medio se suplementó con 50 mg/l de kasugamycin y 200 mg/l de deoxycholate de sodio. Se probó la detección de X. albilineans en un total de 198 tallos; 100 de los cuales fueron asintomáticos y 98 sintomáticos. Las determinaciones se hicieron en cinco clones de caña de azúcar. Para evaluar los métodos de aislamiento se compararon las diluciones de extractos de savia de los entrenudos con los cortes superficiales frescos del mismo entrenudo.