Infectivity Titration for Assessing Resistance to Leaf Scald Among Sugarcane Cultivars

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ABSTRACT


Greenhouse experiments were conducted to determine potential of infectivity titration to evaluate resistance of sugarcane to leaf scald disease caused by Xanthomonas albilineans. In two experiments, single-bud cuttings were inoculated with suspensions containing 10^3, 10^5, or 10^8 CFU/ml of X. albilineans. The occurrence of symptoms was recorded every 15 days from 45 to 210 days after inoculation. At the final evaluation date, leaf vascular sap was plated onto selective medium to detect latent infections. ED50 (log10 of the bacterial concentration required to infect 50% of inoculated plants) was estimated for each cultivar based on probit analysis of cumulative infection frequency. Frequency of infected plants varied among inoculum doses and cultivars and resulted in ED50 values ranging from 3.0 to 12.3 and 3.1 to 9.8 in the first and second experiments, respectively. Good agreement between experiments was observed for ED50 values of individual cultivars. Differences in ED50 among cultivars agreed with field observations of natural disease incidence. Cultivar responses to leaf scald were compared based on the cumulative frequencies of death and recovery in symptomatic plants, and the frequencies of symptomatic plants observed at different evaluation dates for plants inoculated with 10^8 CFU/ml of X. albilineans. Good agreement between ED50 values and these responses was observed. Greenhouse inoculation tests using infectivity titration or just one inoculum concentration could provide an alternative to field tests for the assessment of sugarcane resistance to leaf scald.

Additional keyword: Saccharum

MATERIALS AND METHODS

Source of sugarcane. In the first experiment, stalks of 13 cultivars (L 62-96, CP 65-357, CP 70-321, CP 72-370, CP 74-383, CP 79-318, LCP 82-89, LH 83-153, LCP 85-384, HoCP 85-845, LCP 86-454, CP 88-739, L 88-63) were collected from plants derived from meristem culture and grown at the Louisiana State University Agricultural Center Experiment Station at St. Gabriel, the USDA-ARS Sugarcane Research Unit in Houma, and a commercial seedcane farm in Theriot, LA. In the second experiment, L 62-96, HoCP 85-845, L 88-63, and CP 88-739 were not included. The basal internode of each stalk was assayed for RSD by alkaline-induced metaxylem autofluorescence (2) of stem cross sections, and confirmed by dark-field microscopic observation of centrifuged plant sap (7) for the presence of C. xyli subsp. xyli. Approximately 120 single-bud cuttings, 4 cm long, were cut from RSD-free stalks of each cultivar for inoculation.

Inoculum preparation. X. albilineans was isolated from cultivar LCP 82-89 as previously described (16). A leaf showing symptoms of leaf scald was washed thoroughly with tap water and dried with a paper towel. Segments approximately 1 cm in length were excised from longitudinal white stripes, transferred to a microscope slide containing sterile water, and observed under a dissecting microscope for exudate containing bacteria. Plant tissue exudate was collected with a Pasteur pipet, transferred to a semi-selective medium (3), and incubated for 5 days. An individual colony, designated XaT, was transferred to a 500-ml flask containing 250 ml of Wilbrink’s broth (17) and grown in shake culture for 3 days at room temperature. Just before inoculation, the broth culture was serially diluted in 1.5 ml microfuge tubes, spread on Wilbrink’s agar and incubated for 5 days at 28°C to estimate inoculum concentration. Dilutions used as inoculum contained approximately 10^7, 10^5, or 10^3 CFU/ml. Isolate Xa IT was used in both experiments.

Inoculation. Single-bud cuttings of each cultivar were immersed in 1 liter of each inoculum suspension in plastic ziplock bags. After 1 h at room temperature, the inoculum was removed and the bags were sealed and maintained overnight at room temperature (9). The next day, the cuttings were moved to the greenhouse and subjected to the experiment. Growth conditions were maintained at 28°C with a 14-h photoperiod. After 15 days, the cuttings were transplanted as described above.
Sterile twigs were used to remove leaf segments from the tubes. Sap volumes of approximately 20 µl were collected from each sample and plated on a semi-selective medium (3) using sterile wood toothpicks. Plates were maintained at 28°C and observed after 7 days for the presence of small, light yellow and nonmucoidal colonies typical of X. albilineans. The cumulative frequency of infection (total number of symptomatic plants plus latent infections) were analyzed using the probit procedure of SAS (Statistical Analysis System, Cary, NC) to estimate ED_{50} values (log_{10} of the bacterial concentration required to infect 50% of the inoculated plants) (5) for each cultivar.

Cultivar response to leaf scald was also measured based on the cumulative frequency of death or recovery in symptomatic plants at all three inoculum concentrations, and on the cumulative frequency of symptomatic plants inoculated with 10^{8} CFU/ml of X. albilineans recorded at each evaluation date. Plants were considered recovered from leaf scald disease if no white stripes were evident from 150 to 210 days after inoculation. Agreement of ED_{50} values with cumulative frequency of death or recovery of symptomatic plants and the cumulative frequency of symptomatic plants at different evaluation dates were tested by correlation analysis. Correlation analyses were conducted only with cultivars included in both experiments.

### RESULTS

White longitudinal stripes, identical to those observed on leaves of diseased plants under field conditions, were observed as early as 30 days after inoculation in both experiments. Symptoms developed earlier in plants inoculated with the higher inoculum concentration (results not shown).

The frequency of infected plants varied among cultivars and inoculum concentrations (Table 1). Approximately 20% of infected plants (19% in the first and 23% in the second experiment) represented latent infections as determined by bacterial isolation from leaf sap. In the first experiment, the infection frequency ranged from zero (eight cultivars) to 11% (L 62-96) with 10^{1} CFU of X. albilineans, 3 (LCP 85-384) to 97% (HoCP 85-845) with 10^{5} CFU, and 9 (LCP 85-384) to 100% (CP 74-383) with 10^{6} CFU. In the second experiment, the frequency ranged from zero (three cultivars) to 22% (LCP 82-89) with 10^{1} CFU of X. albilineans, 9 (LCP 85-384 and LHo 83-153) to 91% (LCP 82-89) with 10^{5} CFU, and 19 (LCP 85-384) to 100% (LCP 82-89) with 10^{6} CFU.

The different frequencies of infection in both experiments resulted in different estimated ED_{50} values and regression equations describing the log_{10} concentration/probit-response relationship (Table 2). The ED_{50} values ranged from 3.0 (HoCP 85-845) to 12.3 (LCP 85-384), and from 3.1 (CP 74-383) to 9.8 (LCP 85-384) in the first and second experiments, respectively. The slopes of the regression lines describing the inoculum concentration/response relationship varied from 0.16 to 0.98 and from 0.14 to 1.94. Chi-square tests indicated significant differences in the cumulative frequency of latent infections among cultivars and inoculum concentrations (Table 1).

### Table 1. Cumulative frequency of leaf scald in sugarcane plants derived from single-bud cuttings inoculated with three concentrations of Xanthomonas albilineans, as determined by symptom observations and pathogen isolation 210 days after inoculation.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>First experiment</th>
<th>Second experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inoculum concentration (log_{10} CFU/ml)</td>
<td>Inoculum concentration (log_{10} CFU/ml)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>HoCP 85-845</td>
<td>0/32</td>
<td>31/32</td>
</tr>
<tr>
<td>CP 74-383</td>
<td>0/31</td>
<td>29/31</td>
</tr>
<tr>
<td>LCP 82-89</td>
<td>0/20</td>
<td>16/25</td>
</tr>
<tr>
<td>CP 72-370</td>
<td>1/32</td>
<td>12/30</td>
</tr>
<tr>
<td>L 62-96</td>
<td>3/28</td>
<td>13/25</td>
</tr>
<tr>
<td>CP 70-321</td>
<td>0/31</td>
<td>17/30</td>
</tr>
<tr>
<td>LCP 86-454</td>
<td>0/25</td>
<td>9/29</td>
</tr>
<tr>
<td>CP 88-739</td>
<td>0/32</td>
<td>9/32</td>
</tr>
<tr>
<td>LHo 83-153</td>
<td>2/29</td>
<td>7/30</td>
</tr>
<tr>
<td>CP 65-357</td>
<td>0/32</td>
<td>5/32</td>
</tr>
<tr>
<td>CP 79-318</td>
<td>1/31</td>
<td>3/28</td>
</tr>
<tr>
<td>L 88-63</td>
<td>1/32</td>
<td>4/31</td>
</tr>
<tr>
<td>LCP 85-384</td>
<td>0/32</td>
<td>1/32</td>
</tr>
</tbody>
</table>

\( ^a \) n.t. = not tested.

### Table 2. ED_{50} (log_{10} of the bacterial concentration required to infect 50% of inoculated plants) determined by probit analysis of cumulative frequencies of infected sugarcane plants, inoculated with three concentrations of Xanthomonas albilineans in two experiments.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>First experiment</th>
<th>Second experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ED_{50} (95% fiducial limits)</td>
<td>Regression equation ( P &gt; ) Chi-square</td>
</tr>
<tr>
<td>HoCP 85-845</td>
<td>3.0 (2.3, 3.7)</td>
<td>( Y = -2.2 + 0.73X )</td>
</tr>
<tr>
<td>CP 74-383</td>
<td>3.2 (2.4, 3.9)</td>
<td>( Y = -3.2 + 0.98X )</td>
</tr>
<tr>
<td>LCP 82-89</td>
<td>3.9 (2.9, 4.8)</td>
<td>( Y = -2.3 + 0.59X )</td>
</tr>
<tr>
<td>CP 72-370</td>
<td>5.0 (4.5, 6.2)</td>
<td>( Y = -3.3 + 0.60X )</td>
</tr>
<tr>
<td>L 62-96</td>
<td>5.2 (1.9, 7.6)</td>
<td>( Y = -1.0 + 0.20X )</td>
</tr>
<tr>
<td>CP 70-321</td>
<td>5.4 (3.8, 7.1)</td>
<td>( Y = -1.3 + 0.24X )</td>
</tr>
<tr>
<td>LCP 86-454</td>
<td>6.8 (5.2, 9.4)</td>
<td>( Y = -1.7 + 0.24X )</td>
</tr>
<tr>
<td>CP 88-739</td>
<td>7.1 (5.6, 9.5)</td>
<td>( Y = -1.7 + 0.24X )</td>
</tr>
<tr>
<td>LHo 83-153</td>
<td>9.2 (5.3, 24.6)</td>
<td>( Y = -1.5 + 0.16X )</td>
</tr>
<tr>
<td>CP 65-357</td>
<td>10.0 (7.3, 24.1)</td>
<td>( Y = -1.6 + 0.16X )</td>
</tr>
<tr>
<td>CP 79-318</td>
<td>9.6 (6.4, 12.8)</td>
<td>( Y = -1.5 + 0.32X )</td>
</tr>
<tr>
<td>L 88-63</td>
<td>10.3 (9.5, 11.0)</td>
<td>( Y = -2.3 + 0.22X )</td>
</tr>
<tr>
<td>LCP 85-384</td>
<td>12.3 (8.8, 59.3)</td>
<td>( Y = -2.0 + 0.16X )</td>
</tr>
</tbody>
</table>

\( ^a \) 95% fiducial limits not estimated by the SAS statistical program.

\( ^b \) n.t. = not tested.
cated that for most cultivars the probability of response increased significantly as the inoculum concentration increased. In no case did the slope of the regression line reach 2.0. This indicates \textit{X. albilineans} cells acted independently to infect the inoculated sugarcane cuttings (5). Based on the mean ED$_{50}$ values (Table 2), the cultivars may be ranked in decreasing levels of resistance as follows: LCP 85-384, L 88-63, CP 79-318, CP 65-357, LHo 83-153, CP 88-739, LCP 86-454, CP 70-321, L 62-96, CP 72-370, LCP 82-89, CP 74-383, HoCP 85-845.

Cultivar response to leaf scald also was compared based on the cumulative frequencies of death or recovery in symptomatic plants (Table 3). For cultivars CP 74-383 and LCP 82-89, over 50% of the symptomatic plants were killed by \textit{X. albilineans} in the first and second experiments, and no plant recovered from leaf symptoms. For cultivars CP 65-357, CP 70-321, CP 72-370, CP 79-318, CP 88-739, L 62-96, LCP 85-384, HoCP 85-845, LCP 86-454, and LHo 83-153, no symptomatic plants were killed by \textit{X. albilineans} in the first experiment, but 9% and 4% of the symptomatic plants of CP 70-321 and CP 65-357, respectively, died in the second experiment. The frequency of recovery for these cultivars ranged from 2 (LCP 85-845) to 91% (CP 79-318) in the first, and from 6 (CP 72-370) to 87% (CP 65-357) in the second experiment.

ED$_{50}$ values for cultivars were highly correlated in both experiments (Table 4). In the first experiment, significant correlation was observed between ED$_{50}$ and the cumulative frequency of dead plants recorded during the entire period the experiment was conducted, and between ED$_{50}$ and the cumulative frequency of symptomatic plants inoculated with $10^3$ CFU/ml of \textit{X. albilineans} recorded from 90 to 210 days after inoculation. In the second experiment, significant correlation was observed between ED$_{50}$ and cumulative frequencies of death and recovery in symptomatic plants, recorded during the entire period the ex-

### Table 3. Cumulative frequencies of symptomatic plants killed by leaf scald disease or recovered from symptoms at the end of the experiment 210 days after inoculations

<table>
<thead>
<tr>
<th>Parameters</th>
<th>First experiment</th>
<th>Second experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Dead</td>
</tr>
<tr>
<td>HoCP 85-845</td>
<td>45</td>
<td>1</td>
</tr>
<tr>
<td>CP 74-383</td>
<td>60</td>
<td>31</td>
</tr>
<tr>
<td>LCP 82-89</td>
<td>39</td>
<td>25</td>
</tr>
<tr>
<td>CP 72-370</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td>L 62-96</td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>CP 70-321</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>LCP 86-454</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>CP 88-739</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>LHo 83-153</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>CP 65-357</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>CP 79-318</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>L 88-63</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>LCP 85-845</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

* n.t. = not tested.

### Table 4. Correlation of ED$_{50}$ values with cumulative frequencies of death and recovery, and of frequencies of symptomatic plants at different evaluation dates in plants inoculated with the highest concentration of \textit{Xanthomonas albilineans}

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Experiment</th>
<th>ED$_{50}$</th>
<th>Coefficient of correlation$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st</td>
<td>2nd</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1st</td>
<td>2nd</td>
<td></td>
</tr>
<tr>
<td>ED$_{50}$</td>
<td>First</td>
<td>Second</td>
<td>First</td>
</tr>
<tr>
<td></td>
<td>1.00**</td>
<td>0.96**</td>
<td>1.00**</td>
</tr>
<tr>
<td>Recovery frequency$^c$</td>
<td>First</td>
<td>Second</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.46</td>
<td>0.46</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>0.77**</td>
<td>0.71*</td>
<td>0.76**</td>
</tr>
<tr>
<td>Death frequency</td>
<td>First</td>
<td>Second</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-0.76**</td>
<td>-0.66*</td>
<td>-0.74**</td>
</tr>
<tr>
<td></td>
<td>-0.70**</td>
<td>-0.69*</td>
<td>-0.74**</td>
</tr>
<tr>
<td>Cumulative frequency of symptomatic</td>
<td>First</td>
<td>Second</td>
<td></td>
</tr>
<tr>
<td>plants 60 days after inoculation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-0.59</td>
<td>-0.51</td>
<td>-0.58*</td>
</tr>
<tr>
<td></td>
<td>-0.33</td>
<td>-0.36</td>
<td>-0.33</td>
</tr>
<tr>
<td></td>
<td>-0.52</td>
<td>-0.50</td>
<td>-0.52</td>
</tr>
<tr>
<td>Cumulative frequency of symptomatic</td>
<td>First</td>
<td>Second</td>
<td></td>
</tr>
<tr>
<td>plants 90 days after inoculation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-0.73*</td>
<td>-0.63*</td>
<td>-0.71*</td>
</tr>
<tr>
<td></td>
<td>-0.43</td>
<td>-0.46</td>
<td>-0.44</td>
</tr>
<tr>
<td>Cumulative frequency of symptomatic</td>
<td>First</td>
<td>Second</td>
<td></td>
</tr>
<tr>
<td>plants 120 days after inoculation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-0.74*</td>
<td>-0.63*</td>
<td>-0.72*</td>
</tr>
<tr>
<td></td>
<td>-0.61</td>
<td>-0.62</td>
<td>-0.61</td>
</tr>
<tr>
<td></td>
<td>-0.69*</td>
<td>-0.64*</td>
<td>-0.68*</td>
</tr>
<tr>
<td>Cumulative frequency of symptomatic</td>
<td>First</td>
<td>Second</td>
<td></td>
</tr>
<tr>
<td>plants 150 days after inoculation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-0.77**</td>
<td>-0.70*</td>
<td>-0.76**</td>
</tr>
<tr>
<td></td>
<td>-0.66*</td>
<td>-0.68*</td>
<td>-0.66*</td>
</tr>
<tr>
<td>Cumulative frequency of symptomatic</td>
<td>First</td>
<td>Second</td>
<td></td>
</tr>
<tr>
<td>plants 180 days after inoculation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-0.83**</td>
<td>-0.76**</td>
<td>-0.82**</td>
</tr>
<tr>
<td></td>
<td>-0.68*</td>
<td>-0.71*</td>
<td>-0.69*</td>
</tr>
<tr>
<td></td>
<td>-0.81**</td>
<td>-0.77**</td>
<td>-0.80**</td>
</tr>
</tbody>
</table>

$^a$ Some coefficients were significant at 95% (*) and 99% (**) levels of probability.

$^b$ ED$_{50}$, effective dose, or inoculum concentration necessary to cause disease in 50% of the plants, estimated based on cumulative frequencies of infected plants inoculated with $10^3$, $10^4$, or $10^5$ CFU/ml of \textit{X. albilineans}.

$^c$ Plants not showing leaf scald symptoms (white leaf stripes) from 150 days after inoculation to the end of the experiment.

$^d$ Latent infections were determined by pathogen isolation from asymptomatic plants.

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experiment was conducted, and between ED_{50} and the cumulative frequency of symptomatic plants, recorded from 150 to 210 days after inoculation.

DISCUSSION

Traditionally, tests of sugarcane cultivar response to leaf scald have been conducted under field conditions with the decapitation inoculation method (4,12,13,18,21,22). This method contains uncontrolled variables that may affect the results. In some cases, the inoculum consists of juice obtained by crushing severely infected sugarcane stalks with no estimation of inoculum concentration. Different inoculum concentrations may result in different plant responses and/or frequency of infections, as demonstrated in this work, especially for the susceptible and resistant cultivars. Another problem is associated with the quality and quantity of the inoculum used, as pointed out by Valdebenito and Tokeshi (20). Vascular bundles of leaf scald-infected sugarcane plants were found to harbor a mixture of bacterial species (19). These bacteria could interact with X. albilineans, interfering with the process of plant infection and disease development (20). Finally, inoculated plant surfaces are exposed to environmental conditions that vary between experiments.

The use of the decapitation inoculation method to assess leaf scald resistance has not always produced results that match field reactions to leaf scald. In experiments in Australia with 15 cultivars of known field reaction under natural conditions, differences in clone reactions occurred between trials, which were attributed to variable weather conditions (4). It was impossible to separate the intermediate and susceptible groups of cultivars, and one cultivar, which was resistant in the field under natural conditions, was rated as intermediate (4). Leaf scald symptom expression in cultivars in inoculated tests conducted in the field under Louisiana conditions also has been variable (M. P. Grisham and J. W. Hoy, unpublished data). In ten experiments conducted between 1993 and 2000 using the decapitation method to determine susceptibility of breeding clones to leaf scald, inoculation was unsuccessful in four.

The cultivars (11 or 12 of the 13) tested in this study, using infectivity titration, were tested in two field experiments using the decapitation method (M. P. Grisham, unpublished). The reactions of CP 65-357 in the field experiments did not agree with the results of the infectivity titration experiments. CP 65-357 was among the most susceptible in the field experiments while ranking tenth most susceptible (Table 2) in the infectivity titration experiments. Cultivar response-year interaction also was observed in the field experiments. For example, CP 70-321 ranked second, sixth, and seventh most susceptible, and CP 74-383 ranked first, third, and fifth most susceptible among the three field experiments. These results indicate the need for an alternative method to assess cultivar responses to leaf scald that would be more consistent and reliable.

Here we show that the response of sugarcane to the leaf scald pathogen can be assessed by infectivity titration experiments carried out under greenhouse conditions. The fiducial limits indicated that the precision of the estimated ED_{50} values varied among cultivars. Precision was poor when estimated values were above the range of inoculum concentrations used, as would be expected. Nevertheless, good agreement among ED_{50} values between experiments was observed. Good agreement between ED_{50} and cumulative frequency of death and recoveries in symptomatic plants also was shown. Concentrations of approximately 10^3 to 10^4 CFU/ml of X. albilineans would be sufficient to infect 50% of the plants of the susceptible cultivars, such as CP 74-383 and LCP 82-89. Furthermore, 52 to 82% of the symptomatic plants of these cultivars were killed. The most resistant cultivars (LCP 85-384, L 88-63, CP 79-318, CP 65-357, and LHo 83-153) would have required a higher concentration of bacterial cells (by 10^3 to 10^4 CFU/ml) for 50% infection, than would be required for susceptible cultivars. In addition, most symptomatic plants of resistant cultivars recovered from symptoms. HoCP 85-845 was an exception. It had a low estimated ED_{50} value, but diseased plants were not killed by the pathogen. At the same time, no recovery was detected. These results suggest HoCP 85-845 may be tolerant to leaf scald.

The same clone rankings were obtained with the cumulative frequencies of symptomatic plants at the highest inoculum concentration (10^8 CFU/ml), as early as 150 days after inoculation. This suggests that the use of one inoculum concentration, with evaluations based only on symptom expression, would be sufficient to assess cultivar resistance. The use of only one inoculum concentration and evaluation procedure would simplify the technique and allow a larger number of cultivars to be tested. This would be a useful technique for the routine assessment of clone reactions determined as part of a cultivar selection program.

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LITERATURE CITED


