Early Detection of *Leifsonia xyli* subsp. *xyli* in Sugarcane Leaves by Real-Time Polymerase Chain Reaction

M. P. Grisham, Y.-B. Pan, and E. P. Richard, Jr., United States Department of Agriculture—Agricultural Research Service, SRRC, Sugarcane Research Unit, Houma, LA

ABSTRACT

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A real-time, polymerase chain reaction (PCR) assay was developed for detecting *Leifsonia xyli* subsp. *xyli* in sugarcane leaf tissue. Real-time PCR assays were conducted on the youngest, fully expanded leaf of three cultivars collected bi-weekly from field nurseries between 11 April and 19 July 2005. *L. xyli* subsp. *xyli* infection was detected in leaves collected at all sampling dates, including those from 1-month-old plants on 11 April. Assays conducted on older, more rapidly growing plants (28 July and 21 October 2005) indicated that leaf position affects assay efficiency. Conventional PCR was less efficient than real-time PCR for detecting *L. xyli* subsp. *xyli* in leaf tissue. Real-time PCR was used to rank cultivars for susceptibility to *L. xyli* subsp. *xyli* infection based on the relative titer of *L. xyli* subsp. *xyli* in leaves of inoculated, 3- and 4-month-old greenhouse-grown plants. The ranking of cultivars by real-time PCR was in close agreement with the ranking determined by tissue-blot enzyme immunoassay performed on tissue from 7- to 9-month-old stalks.

Sugarcane plants infected with *Leifsonia xyli* subsp. *xyli* (formerly *Clavibacter xyli* subsp. *xyli*) (9,11), the cause of ratoon stunting disease (RSD), produce no characteristic external sign or symptom of the disease. Commonly used immunoassays lack the sensitivity to detect low titer of *L. xyli* subsp. *xyli* in leaf tissue, but are effective for detecting *L. xyli* subsp. *xyli* in stalk tissue of plants more than 5 months old where the bacterial titer is higher. The inability to assay plants prior to stalk formation prevents timely field diagnosis.

L. xyli subsp. *xyli* is transmitted from field to field in systemically infected stalks or cuttings (seed cane) used to establish new fields (17). Within-field spread occurs mechanically, primarily during harvesting operations (21,22). Therefore, the primary strategy for controlling RSD is to establish a new crop with pathogen-free seed cane and to practice effective sanitation procedures to prevent plant-to-plant spread dur-

ing the crop cycle. A crop cycle consists of an annual harvest that begins with a plant-cane crop (first-year crop) followed by multiple ratoon crops.

Options for creating pathogen-free seed cane include heat treatment to eliminate the bacterium (2,15) and the micropropagation of seed cane from foundation plants that test negative for *L. xyli* subsp. *xyli* (20). To minimize the opportunity for *L. xyli* subsp. *xyli* to reinfect healthy cane during planting and harvest, all cutting implements should be disinfested, especially the cutting surfaces of mechanical harvesters (17).

Techniques to diagnose infected plants in seed cane nurseries and production fields are needed to ensure that seed cane has minimal incidence of RSD and that sanitation practices are effective in preventing infection of production fields. Laboratory-based techniques are required for effective diagnosis because *L. xyli* subsp. *xyli* infection does not cause any characteristic external sign or symptom in sugarcane (15). The primary diagnostic techniques used include microscopy, serological tests, host-induced response, and DNA-based methods (17). The appropriate application of the different techniques depends on several factors, including the distribution and titer of the bacterium in the host plant, the use and urgency of the results, quantity of samples for processing, and availability of equipment and skilled labor (17).

L. xyli subsp. *xyli* is a xylem-inhabiting bacterium and, although infection is systemic in the sugarcane plant, the titer of the bacterium varies among the tissues.

Diagnosis typically has focused on the stalk because bacterial titer is highest there. In the stalk, maximum titer is reached first in the basal internode and then in internodes progressively toward the apex of the stalk (18). Reliable and accurate diagnosis is dependent on the sensitivity of the diagnostic protocol, tissue selection, and timing of sample collection (17,22).

The most commonly used technique for monitoring RSD in Louisiana is the tissue-blot enzyme immunoassay (TB-EIA) (8,22). In a comparison of five diagnostic protocols—TB-EIA; evaporative-binding (EB)-EIA (5); dot-blot (DB)-EIA (19); dark-field microscopy (1); and alkaline-induced red autofluorescence (AIMA), a host-induced response (6)—TB-EIA was the most accurate (22).

Several DNA-based diagnostic methods are highly specific and more sensitive than other methods (10,12,26,27,31). However, polymerase chain reaction (PCR) assays and other DNA-based methods applied directly from sap or other plant tissues were much less sensitive than detection in axenic cultures of the bacterium (10,26). Some improvement in efficiency was made by adding specific reagents such as polyvinylpyrrolidone and Ficoll to reduce the effect of PCR inhibitors (12,26), submitting the sap to a freeze-thaw cycle (27), or the separation of bacterial cells from the sap by differential centrifugation (10).

Real-time PCR increasingly is applied to the diagnosis and quantification of plant pathogens (13,23,29,30). Real-time PCR offers increased sensitivity (3,23,24), is sequence specific (27,32), and is less labor intensive than conventional PCR because post-amplification processing is not required (28,32). With real-time PCR, the amplified product is monitored progressively through the incorporation of fluorescent molecules in the reaction. Fluorescence intensity is correlated to PCR product production (32).

In the temperate climate of Louisiana, sugarcane planting occurs when the seed cane is 4 to 6 months old (mid-July to mid-September). To provide growers with RSD diagnosis of potential seed cane, testing for *L. xyli* subsp. *xyli* infection needs to be completed before the start of planting. At this and earlier stages of growth, TB-EIA for RSD diagnosis is inconsistent and inaccurate (M. P. Grisham, *unpublished*). Consequently, a more sensitive diagnostic

Corresponding author: M. P. Grisham
E-mail: mgrisham@srcc.ars.usda.gov

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technique is needed for consistent and accurate early-season detection of *L. xyli* subsp. *xyli*-infected plants. The objectives of this study were to develop a real-time PCR protocol for the *in vivo* detection of *L. xyli* subsp. *xyli*, to test the efficiency of the real-time PCR protocol at different stages of plant growth, and to determine the effectiveness of ranking sugarcane cultivars for susceptibility to *L. xyli* subsp. *xyli* infection at an early stage of plant growth with real-time PCR.

MATERIALS AND METHODS

Sample collection. Sugarcane plants for testing were identified in an RSD nursery at the United States Department of Agriculture, Agricultural Research Service, Sugarcane Research Laboratory research farm in Shreveport, LA. In 2002, to establish plots with RSD-affected plants, single-bud cuttings were dip inoculated in juice extracted from *L. xyli* subsp. *xyli*-infected stalks, germinated in the greenhouse, and transplanted to the field after approximately 4 months of growth. Control plots were established from single-bud cuttings taken from stalks that had been immersed in a 50°C, circulating hot-water bath for 2 h (2).

The youngest fully expanded leaf with a visible dewlap (the collar between the leaf blade and sheath) was collected from eight plants of three cultivars on each sampling date, four from *L. xyli* subsp. *xyli*-inoculated plants and four from the control plots. Cultivars included L 62-96, a highly susceptible cultivar; CP 70-321, an intermediate susceptible cultivar; and CP 52-68, a resistant cultivar (16; M. P. Grisham, unpublished data). The plants were from the second-ratoon crop of the nursery. Samples were collected on 11 and 26 April, 10 and 24 May, 14 and 28 June, and 19 July 2005. On the 19 July sampling date, there was sufficient stalk development to also collect a sample of the most basal, aboveground internode, including the adjacent nodal tissue.

Leaf samples were stored at -20°C until analyzed by conventional and real-time PCR. The stalk samples were processed the same day for TB-EIA analysis following the protocol developed by Harrison and Davis (18).

To determine the effect of collecting samples from leaves at different positions on the stalk, two sets of samples were collected for real-time PCR analysis. On 28 July, leaves were collected from *L. xyli* subsp. *xyli*-inoculated plots of CP 70-321 and L 62-96. In addition to the youngest fully expanded leaf, the next five leaves below it also were collected. The leaves were designated as leaf 1 (the youngest leaf), then leaves 2, 3, 4, 5, and 6, with leaf 6 being the most basal and the oldest. As on 28 July, samples collected on 21 October consisted of six leaves per stalk; however, sample collection was expanded to

include leaves from *L. xyli* subsp. *xyli*-inoculated and hot-water-treated plots of the three test cultivars.

On 9 September, leaf 5 was collected from eight plants of each of the three cultivars (four from *L. xyli* subsp. *xyli*-inoculated and four from control plots) for real-time and conventional PCR assay. Basal internode samples also were collected on 9 September from all treatments for TB-EIA analysis.

Primer design and real-time PCR conditions. Primers were designed using Beacon Designer 2 software (Palo Alto, CA) specifically for real-time PCR and selected to amplify relatively short fragments. Initially, primers were designed within the 438-bp product amplified with the primer set developed by Pan et al. (26) for PCR detection of *L. xyli* subsp. *xyli*. Among the potential primer pairs identified, primers Lxx82F (TTCAACGCA GAAATTGTCCAGG) and Lxx22R (CAAGCAGGCGTACTAAGTTCGA) were selected. They amplified a 139-bp fragment. The optimal annealing temperature was 64°C. For the last two sample collections (9 September and 21 October), another set of primers was designed from the intergenic transcribed spacer (ITS) region of 16S-23S ribosomal DNA of *L. xyli* subsp. *xyli* (GenBank accession number AF056003). The specificity of both primer sets was checked by alignments with GenBank sequences using the standard nucleotide-nucleotide BLAST (blastn; provided online by the National Center for Biotechnology Information). Among the potential primer pairs identified, primers Lxx202F (CGAACTTAGTACGCCTCGTTG) and Lxx331R (GGATTTCGGTTCTCATCTC AGC) were selected. They amplified a 130-bp fragment. The optimal annealing temperature was 60°C. All primers were commercially synthesized by Integrated DNA Technologies (Coralville, IA).

Leaf DNA extraction and real-time PCR amplification. Total DNA was extracted from approximately 200 mg of leaf tissue as described by Pan et al. (25). The real-time PCR reactions were performed in the iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA) and were carried out in 96-well reaction plates. Each reaction mixture contained 1 µl of sample, 7 µl of SYBR green master mix, 0.1 µl of forward primer, 0.1 µl of reverse primer, and 5.8 µl of distilled water. Two sources of SYBR green master mix (iQ SYBR Green Supermix; Bio-Rad Laboratories, Inc., and SYBR Green PCR Master Mix; Applied Biosystems, Foster City, CA) were used with comparable results (*data not shown*). Negative control reactions contained the same mixtures with 0.1 µl of sterile water replacing the sample. Thermal cycling conditions consisted of 5 min at 95°C followed by 40 cycles each consisting of denaturation for 10 s at 95°C, annealing for

30 s at 64°C (primer set Lxx82F/Lxx22R) or 60°C (Lxx202F/Lxx331R), and extension for 10 s at 72°C. After the final amplification cycle, a melting-curve analysis was performed to confirm specific amplification (24,27). The expected product of 139-bp from the first set of primers had a melting temperature (T_m) of 87°C; the second primer pair was expected to produce a 130-bp fragment with a T_m of 86°C. Data analysis was carried out with the manufacturer's software (Optical System Software, v. 3.0; BioRad Laboratories, Inc.). The nucleotide sequences of the PCR products were determined on CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc., Fullerton, CA) following the manufacturer's protocol. Conventional PCR was conducted using primers and thermocycle program as described by Pan et al. (26).

Assessing cultivar resistance. Stalks of nine sugarcane cultivars were hot-water treated (2) as described above to eliminate *L. xyli* subsp. *xyli* infection, and then cut into single-bud sets. One group of single-bud cuttings of each cultivar was dip inoculated with juice extracted from *L. xyli* subsp. *xyli*-infected stalks, planted in 15-cm-diameter pots filled with potting soil, and placed in the greenhouse, and another group of cuttings was planted without inoculation. Three months after inoculation, three plants from inoculated buds and three from noninoculated buds were selected for the study within each cultivar. Selected plants were similar in size and development.

The youngest, fully expanded leaf was collected from each plant, total DNA was extracted, and real-time PCR amplification was performed as described above. Cultivars were ranked for susceptibility to *L. xyli* subsp. *xyli* infection based on their average threshold cycle (Ct) values. The lower the Ct value for a cultivar, the higher the number of bacteria in the tissue sample (32). The real-time PCR analysis was repeated 1 month later (4 months after inoculation).

Ranking of cultivar susceptibility to *L. xyli* subsp. *xyli* infection based on real-time PCR analysis was compared with ranking based on percent vascular bundles colonized (CVB) determined with TB-EIA (18). The TB-EIA was performed on four 7- to 9-month-old stalks of each cultivar collected annually from the plant-cane, first-ratoon, and second-ratoon crops of the RSD nursery established in 2002. A longitudinal core from the lowest aboveground internode of each stalk was blotted on a nitrocellulose membrane, the membrane stained with a labeled antibody, and the percent CVB determined.

RESULTS

All leaf samples of the highly susceptible cv. L 62-96 collected between 11 April and 19 July from *L. xyli* subsp. *xyli*-

inoculated plots were diagnosed as positive for *L. xyli* subsp. *xyli* infection using real-time PCR (Table 1). Six leaf samples from the hot-water-treated plots of L 62-96 also tested positive (Table 1). For the intermediate susceptible cultivar CP 70-321, all leaves from the *L. xyli* subsp. *xyli*-inoculated plots tested positive for *L. xyli* subsp. *xyli* infection with real-time PCR, except for the first two sampling dates and the 19 July date, whereas only one sample from the hot-water-treated plot tested positive (Table 1).

For the first four sampling dates, diagnosis with conventional PCR indicated that all samples of L 62-96 were positive for *L. xyli* subsp. *xyli* infection, in agreement with the real-time PCR results (Table 1). The proportion of samples testing positive, however, declined to 75, 50, and 0% with conventional PCR on the last three sampling dates, respectively. Only about 23% of the samples testing positive among the leaves collected from the *L. xyli* subsp. *xyli*-inoculated plots of CP 70-321 by real-time PCR tested positive by conventional PCR (Table 1). No sample from a hot-water-treated plot of L 62-96 or CP 70-321 tested positive with conventional PCR. No sample collected from *L. xyli* subsp. *xyli*-inoculated or hot-water-treated plots of CP 52-68 (*L. xyli* subsp. *xyli* resistant) tested positive with real-time or conventional PCR for the April 11 to July 19 sampling dates (*data not shown*).

Sufficient stalk tissue to employ TB-EIA for *L. xyli* subsp. *xyli* infection testing did not develop until 19 July. All four samples of L 62-96, three of four samples of CP 70-321, and none of CP 52-68 from *L. xyli* subsp. *xyli*-infected plots tested positive for *L. xyli* subsp. *xyli* infection. The L 62-96 samples from the *L. xyli* subsp. *xyli*-infected plots were also positive with the real-time PCR; however, only one CP 70-321 sample was positive for *L. xyli* subsp. *xyli* infection with real-time PCR (Table 1).

Plants infected with *L. xyli* subsp. *xyli* were detected by real-time PCR on each sampling date; however, a decline in the proportion of positive assays occurred across all samples, from an average of 42% of the samples positive over the first five sampling dates to 31 and 19% on the sixth (28 June) and seventh (19 July) sampling dates, respectively (Table 1). The youngest leaf with a visible dewlap was chosen for analysis because it is an easily identifiable tissue source and, as such, provides a degree of consistency among samples collected from different cultivars, at different growth stages, and by different collectors. Real-time PCR assay of the youngest, fully expanded leaf and the five leaves basal to it collected on 28 July from *L. xyli* subsp. *xyli*-infected plots of L 62-96 and CP 70-321 showed that the largest number of positive samples was those from leaf number 5 (Table 2).

When the first set of primers (Lxx82F/Lxx22R) was developed, they appeared to meet the guidelines provided by the manufacture of the iCycler iQ Real-Time PCR Detection System (Bio-Rad

Laboratories, Inc.), including absence of nonspecific products such as primer-dimers. Testing of the primer design was conducted using samples containing high numbers of target DNA (high numbers of *L. xyli* subsp. *xyli* bacteria); however, under conditions of low titer of the target DNA, melt curve analysis indicated the coamplification of a nonspecific product (T_m of 78°C) with the specific product (T_m of 87°C). Gel analysis confirmed that the larger real-time PCR product was 139 bp, corresponding to the expected size of the specific product.

As recommended when nonspecific products are produced, a new primer set was designed for detecting *L. xyli* subsp. *xyli* (Lxx202F/Lxx331R) that produced a single melting peak, a single band of expected size on the electrophoresis gel, and the expected nucleotide sequence (24). This primer set was used for the samples analyzed on 9 September and 21 October (Tables 2 and 3). A number of samples collected between 11 April and 19 July were reanalyzed using the redesigned primers. Earlier diagnosis was confirmed

Table 2. Effect of leaf position as source of tissue for diagnosis of *Leifsonia xyli* subsp. *xyli* infection in a sugarcane plant as determined by real-time polymerase chain reaction

Collection date, cultivar	Source ^b	Leaf position ^a					
		1	2	3	4	5	6
28 July							
L 62-96	RSD	3	4	4	4	4	4
CP 70-321	RSD	1	1	1	1	4	3
21 October							
L 62-96	RSD	4	4	4	4	4	4
	HWT	1	2	2	3	2	2
CP 70-321	RSD	0	2	2	1	3	2
	HWT	0	0	0	0	0	0
CP 52-68	RSD	0	0	1	0	0	0
	HWT	0	0	0	0	0	0

^a Position 1 = the youngest, fully expanded leaf with a visible dewlap; 2 = leaf immediately basal to leaf 1; 3 = leaf immediately basal to leaf 2; and so on. Data indicate number of plants positive for *L. xyli* subsp. *xyli* infection out of four plants assayed.

^b Nursery source. RSD = samples collected from *L. xyli* subsp. *xyli*-inoculated nursery plot, and HWT = samples collected from hot-water-treated nursery plot.

Table 1. Comparison of diagnostic techniques for detecting *Leifsonia xyli* subsp. *xyli* infection of sugarcane^a

Date	LCP 62-96 (susceptible)				CP 70-321 (intermediately susceptible)				Positive (%) ^b
	Real-time PCR		Conventional PCR		Real-time PCR		Conventional PCR		
	RSD	HWT	RSD	HWT	RSD	HWT	RSD	HWT	
April									
11	4 ^c	0	4	0	2	1	0	0	34
26	4	2	4	0	3	0	1	0	44
May									
10	4	1	4	0	4	0	2	0	47
24	4	0	4	0	4	0	1	0	41
June									
14	4	2	3	0	4	0	1	0	44
28	4	0	2	0	4	0	0	0	31
July									
19	4	1	0	0	1	0	0	0	19

^a Analysis performed on total DNA extracted from tissues of the youngest, fully expanded leaf with a visible dewlap using cetyltrimethylammonium bromide extraction buffer procedure. PCR = polymerase chain reaction, RSD = samples collected from *L. xyli* subsp. *xyli*-inoculated nursery plot, and HWT = samples collected from hot-water-treated nursery plot.

^b Positive for *L. xyli* subsp. *xyli*.

^c Each value is the number of samples testing positive for *L. xyli* subsp. *xyli* infection out of four samples tested.

without the production of nonspecific products (*data not shown*).

Among the four leaf samples of each cultivar collected on 9 September, four samples of L 62-96, three of CP 70-321, and two of CP 52-68 from *L. xyli* subsp. *xyli*-inoculated plots tested positive for *L. xyli* subsp. *xyli* infection when analyzed with real-time PCR utilizing the redesigned primer set (Table 3). One L 62-96 and one CP 70-321 sample from the hot-water-treated plots tested positive for *L. xyli* subsp. *xyli* infection. No sample tested positive when analyzed with conventional PCR. TB-EIA analysis also indicated that the L 62-96 sample from the hot-water-treated plot was positive; however, the CP 70-321 sample from the hot-water-treated plot and the two CP 52-68 samples from the *L. xyli* subsp. *xyli*-inoculated plots tested negative with the TB-EIA assay (Table 3). All samples of L 62-96 and CP 70-321 from the *L. xyli* subsp. *xyli*-inoculated plots tested positive with TB-EIA.

The ranking of the nine cultivars for susceptibility to *L. xyli* subsp. *xyli* infection using real-time PCR was similar for the two sampling dates (Table 4), and the ranking by real-time PCR also was in close agreement with the ranking determined by TB-EIA.

DISCUSSION

Real-time PCR was effective in detecting *L. xyli* subsp. *xyli* infections at all stages of plant growth, including early in the season, when leaves were the only aboveground tissues available for sampling and assaying. The ability to collect diagnostic samples earlier in the season before the plant lodges improves sampling coverage because of better access to fields. Coverage of fields also can be improved by pooling leaf samples for real-time PCR diagnosis. If a pooled sample tests positive, the diagnostician has the option to separate the samples and run them individually.

Growth of test plants was initiated approximately 4 weeks prior to the first sampling date; however, the growth rate was slow because of cool weather and soil temperatures. The lower detection level observed in cv. CP 70-321 for the earliest sampling dates (Table 1) suggests that detectable titer levels in leaves of intermediately susceptible cultivars may not be found until more active growth is sustained.

TB-EIA is limited to sampling after the formation of stalk tissue. For most cultivars grown in Louisiana, stalks with one or more fully expanded internodes do not occur until late June or early July. In this study, stalk tissue was not available to perform TB-EIA until the 19 July sampling date. Also, in collecting stalk tissue for TB-EIA or other techniques that require a high titer of bacteria found in the vascular

sap of the mature internodes, the stalk must be cut. The loss of a few stalks in a seed cane or production field is not critical; however, destructive sampling can be a problem in some situations. For example, a nondestructive sampling would be useful for the RSD diagnosis of critical foundation plants used in micropropagation operations or when indexing plant material in quarantine programs.

Gillaspie (14) developed a nondestructive method of RSD diagnosis. Basal parts of the oldest leaves were centrifuged at low speed and the centrifugal fluid then was examined by phase-contrast microscopy for the presence of *L. xyli* subsp. *xyli* bacterial cells. He reported detection of *L. xyli* subsp. *xyli* in some samples collected as early as 30 April. The limitation of this method is the effort required to set up the centrifugation and the labor and skill needed for the microscopic examination of the sample. The study did establish that *L. xyli* subsp. *xyli* bacterial cells could be recovered from leaves of young plants.

The samples that tested positive for *L. xyli* subsp. *xyli* infection by conventional PCR also tested positive by real-time PCR (Table 1); however, real-time PCR was

more effective in identifying *L. xyli* subsp. *xyli*-infected plants in the intermediately susceptible cv. CP 70-321 and in the resistant cv. CP 52-68 and among samples from later sampling dates and from hot-water-treated plots. Pan et al. (26) improved the sensitivity of the conventional PCR protocol when analyzing juice from stalks with the addition of polyvinylpyrrolidone and Ficoll to reduce the effects of PCR inhibitors. These were not included in the protocol used in this study because, in preliminary studies that included only early growth stage-infected leaves of the highly susceptible cv. LCP 62-96, their use did not appear necessary. In future comparisons of real-time PCR and conventional PCR, these materials should be added to the conventional PCR reaction buffer. Even with increased sensitivity of conventional PCR, an advantage of real-time PCR is that no post-reaction analysis of the PCR products on gel electrophoresis is required, resulting in reduced experimental time and increased throughput.

The youngest, fully expanded leaf appeared to be a good source of tissue for diagnosis in plants prior to the development of stalks; however, after stalk devel-

Table 3. Comparison of diagnostic techniques for detecting *Leifsonia xyli* subsp. *xyli* infection in leaf 5 of approximately 7-month-old sugarcane stalks (9 September 2005)^a

Cultivar, source ^b	Real-time PCR	Conventional PCR	TB-EIA
L 62-96			
RSD	4	0	4
HWT	1	0	1
CP 70-321			
RSD	3	0	4
HWT	1	0	0
CP 52-68			
RSD	2	0	0
HWT	0	0	0

^a Leaf 5 is the fourth leaf basal to leaf 1 (the youngest, fully expanded leaf with a visible dewlap). PCR = polymerase chain reaction and TB-EIA = tissue-blot enzyme immunoassay. Data indicate number of plants positive for *L. xyli* subsp. *xyli* infection out of four plants assayed.

^b Nursery source. RSD = samples collected from *L. xyli* subsp. *xyli*-inoculated nursery plot, and HWT = samples collected from hot-water-treated nursery plot.

Table 4. Comparison of real-time polymerase chain reaction (PCR) and tissue-blot enzyme immunoassay (TB-EIA) for ranking nine sugarcane cultivars for susceptibility to *Leifsonia xyli* subsp. *xyli* infection

Real-time PCR				TB-EIA	
3-month-old plant		4-month-old plant			
Cultivar	Mean Ct ^a	Cultivar	Mean Ct ^a	Cultivar	Mean CVB (%) ^b
Ho 95-988	—	Ho 95-988	—	Ho 95-988	2
CP 52-68	37.4	LCP 85-384	38.3	CP 52-68	5
HoCP 96-540	35.5	CP 52-68	37.4	HoCP 96-540	9
LCP 85-384	35.0	HoCP 96-540	30.8	LCP 85-384	23
L 99-226	27.7	L 99-226	28.6	CP 70-321	41
CP 70-321	26.0	CP 70-321	27.4	L 99-226	69
HoCP 00-950	25.8	HoCP 00-950	27.7	HoCP 00-950	77
L 99-233	24.8	L 99-233	26.0	L 99-233	86
L 62-96	23.5	L 62-96	25.9	L 62-96	94

^a Threshold cycle (Ct) number when fluorescence of sample exceeded background fluorescence.

Lower Ct number indicates fewer PCR cycles necessary for detection because of the presence of a greater number of *L. xyli* subsp. *xyli* bacteria in sample. The greater the Ct number, the more resistant the cultivar; n = 3; — = fluorescence of sample did not exceed background.

^b CVB = colonized vascular bundles; n = 12.

opment was initiated, the choice of older, more mature leaves may improve efficiency of the RSD diagnosis. The reduced efficiency of detecting *L. xyli* subsp. *xyli* by conventional PCR in L 62-96 also suggests that, at the older stages of plant growth, PCR inhibitors also build up in leaves (Table 3).

The detection of *L. xyli* subsp. *xyli* among samples collected from the hot-water-treated (control) plots created some initial concern. Factors that may explain *L. xyli* subsp. *xyli* infection among the control plants of L 62-96 include an incomplete eradication of *L. xyli* subsp. *xyli* during the hot-water treatment of the seed cane used to plant the control plots or the infection of the control plot during the two earlier harvests of the nursery. Although hot-water treatment of seed cane is highly effective at reducing infections of *L. xyli* subsp. *xyli*, complete elimination of the pathogen may not always occur (2,7,15). The plants used in this study were from a second-ratoon nursery. As much care as possible was taken to prevent mechanical spread of *L. xyli* subsp. *xyli* to the control plots, including disinfecting the harvester cutting blades and harvesting the control plots first. With a highly susceptible cultivar such as L 62-96 that also supports a high titer of bacteria, potential for accidental spread of *L. xyli* subsp. *xyli* is higher than for other, less susceptible cultivars (22).

The ranking of cultivars for susceptibility to *L. xyli* subsp. *xyli* infection was in close agreement with the ranking determined by TB-EIA, a reliable and often-used method to assess cultivar susceptibility to RSD (4,8,18,20,21). Currently in Louisiana, resistance to RSD is not a primary selection criterion for advancing candidate cultivars because an efficient method to evaluate clones at the early stages of the breeding program is not available and because the disease can be controlled with the use of pathogen-free seed cane and field sanitation. Real-time PCR offers the advantage that testing for resistance to RSD can be performed on plants prior to the development of mature stalks. Therefore, evaluations can be made in a timelier manner, and the number of cultivars that can be efficiently evaluated can be increased.

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