Method for Detecting *Clavibacter xyli* subsp. *xyli* from Sugarcane Leaves

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ABSTRACT

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Clavibacter xyli subsp. *xyli*, the causal agent of ratoon stunting disease, was detected in centrifugal extracts from sugarcane leaves that had been infiltrated with water. The basal parts of the oldest green leaf blades were cut in 2.5-cm sections, vacuum-infiltrated with water, and centrifuged at low speed. Leaf sections were elevated in the centrifuge tube with a plastic support, or enough leaves were lodged in the microfuge tube to prevent them from moving into the centrifugal fluid. Extracts were examined by phase-contrast microscopy. This procedure provides a method to detect the pathogen without destruction of the shoot.

Additional key words: *Saccharum* interspecific hybrids

The causal agent of ratoon stunting disease (RSD) of sugarcane (*Saccharum* interspecific hybrids) was described as a small, coryneform bacterium measuring 0.2× 1–10 μm (4) and was characterized taxonomically as *Clavibacter xyli* subsp. *xyli* Davis et al (5). Diagnosis of RSD is difficult because there are no specific external symptoms in sugarcane and internal symptoms do not develop adequately in all clones (9). Diagnostic methods include infectivity assays (7,9,12), serological techniques (3), and electron microscopy (13) and phase-contrast microscopy of stalk extracts (2,6,11). In these methods, the tested shoot must be cut. Because no nondestructive assay has been developed, the possible use of leaves for the detection of the bacterium was investigated.

The bacterium is distributed systemically in sugarcane plants (1,10,13). Tissue pieces from diseased or suspect plants were soaked in water for 1.5–3 hr, and bacteria, which oozed into the water, were concentrated by centrifugation. Problems with this procedure are that at least 3 hr per sample was needed for processing before examination by phase-contrast microscopy, and recovery of RSD-bacteria from diseased plants was inconsistent for the three clones tested by Bailey (1).

In 1965, Klement (8) reported an infiltration-centrifugation method to obtain fluids from the intercellular spaces

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of foliage. His method involves vacuum-infiltrating tobacco leaves, placing them in a centrifuge tube with a screen support to prevent the leaf from moving into the centrifugal fluid, and centrifuging at 1,120 × *g* for 10 min. The research described here was initiated to evaluate the applicability of the Klement method for the detection of *C. x.* subsp. *xyli* in sugarcane leaves.

MATERIALS AND METHODS

Sugarcane plants for these evaluation experiments were grown in field nurseries of the U.S. Sugarcane Field Laboratory near Houma, LA. Plots of RSD-infected and heat-treated (hot water, 50 C, 2 hr) seed canes had been planted in October 1984. Samples were removed from the plants at various dates, inserted in polyethylene bags, and shipped to Beltsville, MD, for analysis for the presence of *C. x.* subsp. *xyli*. All samples were coded in Louisiana before shipment so that the analyses would not be biased. These shipments were in transit for 3–5 days.

In Beltsville, the leaf sheath was removed, and the basal portion of the leaf blade (lamina and midrib) was cut into 2.5-cm sections, vacuum-infiltrated with distilled water, and blotted on paper toweling to remove excess water. Comparable sections of the leaves were combined for extraction. For the clinical centrifuge method of fluid extraction, the leaf sections were placed into round-bottom centrifuge tubes (50 ml), each containing a perforated plastic disk that held the section above the tube bottom. Leaf sections were positioned so that the midribs were parallel to the sides of the tube. Samples were centrifuged at 600 × *g* for 5 min. The leaf sections were removed with forceps, and the plastic disk was removed with a bent dissecting needle. The centrifugal fluid that had collected below the plastic disk was removed with a Pasteur pipette, and the samples were stored frozen at −20 C until they were examined by phase-contrast microscopy.

A microfuge also was used to extract fluid from water-infiltrated sugarcane leaves. Extracts were collected by placing sections (1–1.25 cm) of infiltrated basal midribs in conical centrifuge tubes (1.5 ml) and centrifuging them at 15,000 × *g* for 1 min in a microfuge. A sufficient number of midrib sections were placed in each tube so that they did not move down into the centrifugal fluid. After centrifugation, the sections were removed with forceps, the fluid was withdrawn with a Pasteur pipette, and the samples were stored frozen at −20 C.

Care was exercised when selecting leaf sections for extraction to avoid leaves with obvious mechanical injury or those with disease symptoms. This was done to prevent introduction of large amounts of contaminant bacteria or fungi that could have hindered detection of *C. x.* subsp. *xyli*.

In the initial tests, the leaves were harvested from 38 clones in an RSD nursery. Each sample consisted of the oldest green leaf of three plants of a clone. No heat-treated control samples were used in these tests, and only the clinical centrifuge extraction method was used.

In further evaluation experiments, comparisons were made between leaf and stem tissue sampling to determine the reliability of using only leaf tissue. For these tests, the two oldest green leaves from infected and control plants were harvested and analyzed. These leaves were extracted by both the clinical centrifuge and the microfuge methods. For the 8 August sample, stalk sections that had been divided into one-node segments were also sent, but only a few of these arrived in good condition. Thereafter, a two-node stalk section from the base of each shoot from which leaf samples had been taken was sent with the leaf samples on 30 September and 13 November. After the nodes were removed, freshly cut internodes were centrifuged in a conical centrifuge tube (50 ml) at 1,000 × *g* for 1 min and the centrifugal fluids were collected. The centrifugal fluid samples were stored frozen at −20 C until they were examined by phase-contrast microscopy for cells of *C. x.* subsp. *xyli*.

After each fluid sample had been thawed and thoroughly mixed, a 10-μl drop was placed on a glass slide and covered with a coverslip 22 × 22 mm. Counts were made of the diagnostic bacteria in 10 microscopic fields viewed under phase contrast at ×1,250. Cells of *C. x.* subsp. *xyli* are easily distinguished from other bacteria or cell debris by their characteristic size and shape (4,6). The coryneform bacterium has a smooth cell wall, is rod-shaped and sometimes bent, and measures 0.25–0.5 × 1–4 μm; 10 μm or longer lengths are not uncommon.

RESULTS AND DISCUSSION

In the initial tests, cells of *C. x.* subsp. *xyli* were detected in extracts from 28 of 38 clones evaluated on April 30 (Table 1). By 10 July, seven additional clones were found to have bacteria; however, one clone, Co 740, remained free of detectable bacteria. The other two clones, POJ 36M and Q 71, were not tested after April because of an oversight.

In further evaluation experiments, samples were taken from both hot-water-treated and RSD-infected clones in August, September, and November. The data collected on CP 70-321 on 30 September using both the clinical and microfuge centrifugation methods for extracting samples and the clinical centrifuge method for corresponding stalk material are presented in Table 2. The bacterium was detected in all of the leaf and stalk extracts from the RSD plot with the exception of one leaf sample. Three of the leaf samples from the hot-water-treated plot also were positive; two of the three corresponding stalk extracts contained diagnostic bacteria, and the other may have contained bacteria. Three additional stalk extracts from the hot-water-treated plot contained *C. x.* subsp. *xyli* at low concentrations, whereas none were detected in the corresponding leaf samples. Results similar to those of the 30 September test were obtained for CP 70-321 and CP 72-370 on 26 August and for CP 65-357 on 13 November. Table 3 summarizes the data from the three evaluation dates to show the reliability of using the leaf assay. Fewer diagnostic bacteria were recovered from leaves in the November test than from the stalk samples, possibly because younger leaves were used for the test since older leaves had been damaged by a hurricane. In preliminary tests of leaf samples from greenhouse-grown sugarcane in Beltsville, the relative

Table 1. Detection of *Clavibacter xyli* subsp. *xyli* in sugarcane clones from a ratoon stunting disease nursery (Houma, LA) by the leaf assay method[a]

Clones	1985 Harvest date	
	30 April	10 July
Cl 41-223, CP 44-101, CP 53-1, CP 55-30, CP 48-103, CP 36-105, CP 36-13, CP 52-1, CP 65-357, CP 67-412, CP 72-370, CP 70-330, Co 281, Co 285, Co 453, L 62-96, L 65-69	+[b]	+
Cl 47-83, CP 45-184, CP 61-37, CP 73-351, CP 72-356, Co 331, CR 63-211, F 36-819, N 53-216, POJ 234	+	NT
CP 52-68, CP 70-321, IAC 50-218, L 60-25, Q 28	−	+
CB 41-76,[c] Chunnee,[c] POJ 36M, Q71	−	NT
CP 29-116	+	−
Co 740	−	−

[a] Sampling by centrifugation (600 × *g*, 5 min) of water-infiltrated leaf sections from the basal portion of the oldest green leaf combined from three plants of each clone. Diagnosis based on examining 10 microscopic fields with a phase-contrast microscope (×1,250).
[b] + = Diagnostic bacteria observed, − = no diagnostic bacteria detected, and NT = not tested.
[c] Positive when retested on 16 May 1985.

bacterial concentrations in infiltrated-centrifuged fluid samples was lower from younger leaves than from older leaves. Generally, the relative concentrations of bacteria in these leaf samples were lower (2–10×) than in samples obtained from internodes just below the point of attachment of the leaves.

Leaf samples that had been in transit for a number of days or that were somewhat dry required vacuum-infiltration with water for good extraction of bacteria. In preliminary tests with fresh green-leaf samples directly from the greenhouse, bacteria could be extracted by centrifugation of leaves without infiltration with water. Omission of the infiltration step would save time if the leaf samples were relatively fresh. This would become important especially when large numbers of leaf samples were evaluated.

The leaf assay method adapted from the Klement method (8) works well for sugarcane. Sugarcane leaves are stiff enough to remain erect and may be handled more easily than the softer, more succulent leaves of tobacco. However, the volume of fluid extract from sugarcane tends to be only one-fifth to one-eighth that collected from tobacco leaves. And the bacterial numbers in the sugarcane leaf samples do not equal those in stalk samples used in other methods (3).

Although bacterial populations extracted from leaf samples were not as large as those extracted from cane, this detection method offers a practical and reliable nondestructive method to determine the incidence of RSD on field or greenhouse plants and possibly in young field-grown plants. The diagnostic bacteria were found in leaf extracts from highly susceptible clones 1 mo after planting in the greenhouse. Early, nondestructive detection can be important for indexing plant material in quarantine programs and research projects, in the early assessment of seed cane nursery plots, and in making decisions on the relative resistance of clones. In addition, the method relies on simple and relatively inexpensive equipment of a type usually available in laboratories for locations working on sugarcane research worldwide.

In the further studies with this method, two factors will need to be explored more fully. One is the relationship between the number of bacteria in the stalk and the number in the leaves. It may well be that tolerant cultivars, with low numbers of bacteria in the stalk, might have so few bacteria in the leaves that they cannot be detected (CP 29-116, CP 52-68, L 60-25). Second, the buildup of bacterial numbers over time may well be a clonal characteristic; the bacterial populations in some clones increase early (CP 72-356), whereas in other clones, population increase is delayed (CP 70-321).

Table 2. Numbers of *Clavibacter xyli* subsp. *xyli* (cells per 10 microscope fields) detected by three methods in samples of CP 70-321 (30 September 1985)

Hot-water-treated			Ratoon stunting disease		
Leaf		Stalk	Leaf		Stalk
Clinical[a]	Microfuge[b]	Clinical[c]	Clinical[a]	Microfuge[b]	Clinical[c]
0	0	2?	4	22	66
1	2	1?	3	10	88
0	0	1	3	3	56
0	0	1	2	1	11
0	0	0	3	9	68
0	0	0	3	14	130
4	1	5	0	1	6
0	0	0	4	2	80
0	0	0	3	11	258
1	3	3	4	1	100

[a] Samples collected by centrifuging in a clinical centrifuge at 600 × *g* for 5 min.
[b] Samples collected by centrifuging in a microfuge at 15,000 × *g* for 1 min.
[c] Samples collected by centrifuging a lower internode in a clinical centrifuge at 1,000 × *g* for 1 min.

Table 3. Comparison of leaf and stalk assays of field-grown shoots from Louisiana to detect *Clavibacter xyli* subsp. *xyli*[a]

Harvest date (1985)	Clone	Nursery	Results for leaf and stalk samples[b]			
			Agree		Disagree	
			Stalk+[c] leaf+	Stalk− leaf−	Stalk+ leaf−	Stalk− leaf+
26 Aug.[d]	CP 70-321	RSD[e]	3	1	2	0
		HWT	1	2	0	0
26 Aug.[d]	CP 72-370	RSD	2	1	1	0
		HWT	3	1	4	0
30 Sept.	CP 70-321	RSD	9	0	1	0
		HWT	2	4	2	0
13 Nov.	CP 65-357	RSD	8	0	1	0
		HWT	1	7	0	0

[a] Based on clinical centrifuge data.
[b] Only results that were clearly positive or negative were used here.
[c] + = Bacteria observed in sample; − = no bacteria observed in sample.
[d] Some stalk samples yielded no centrifugal fluid.
[e] RSD = ratoon stunting diseased nursery; HWT = hot-water-treated nursery.

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