Comparison of Diagnostic Techniques for Determining Incidence of Ratoon Stunting Disease of Sugarcane in Florida

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ABSTRACT

Davis, M. J., and Dean, J. L. 1984. Comparison of diagnostic techniques for determining incidence of ratoon stunting disease of sugarcane in Florida. Plant Disease 68:896-899.

Fluorescent-antibody staining was more accurate for detecting the RSD bacterium in xylem sap extracts from sugarcane than was phase-contrast microscopy or isolation in culture. Concentration of fluorescent antibody-stained bacteria on the surface of polycarbonate membrane filters (0.2-μm pore-size) by filtration before examination by epifluorescent microscopy resulted in a sevenfold to eightfold increase in sensitivity of detection compared with the usual method of staining dried sap samples on the surface of microscope slides. When phase-contrast microscopy, isolation in culture by the dilution-plate technique, and fluorescent-antibody stains on membranes (FASM) were compared, 95 of 120 (79%) samples from 20 commercial sugarcane fields were determined to be infected with the ratoon stunting disease (RSD) bacterium by at least one of these diagnostic techniques, and about 20% more infections were detected by FASM than by the other two techniques. The frequency of detection of the RSD bacterium in the commercial fields by FASM was 18% greater on 26 October 1982 than on 1 September 1982, with 53 of 60 (88%) samples having bacteria in October. Only plant crops were examined and the RSD bacterium was detected in plants from all fields. Both incidence of RSD and the average population of the RSD bacterium in sap extracts varied among sugarcane clones.

On a worldwide basis, ratoon stunting disease (RSD) of sugarcane (interspecific hybrids of *Saccharum*) may be responsible for more yield loss than any other disease of sugarcane (13). Ratoon stunting is a vascular disease caused by an extremely small, xylem-inhabiting, coryneform bacterium (2,7,22). The only external symptom of the disease is stunting, which alone is not characteristic of RSD. Vascular discoloration, an internal symptom, has been used in diagnosis, but this symptom may be ephemeral or absent in some clones (10,20). Thus, the incidence and, consequently, the importance of RSD in commercial production is difficult to assess.

In areas of the world where sugarcane is periodically subjected to water stress, yield losses caused by RSD may occur in clones otherwise tolerant to the disease (19). Heat treatment and use of RSD-tolerant clones is often practiced for RSD control where losses caused by RSD are readily noticeable (20). Because of the unique system of regulating the water table in the Everglades agricultural region of Florida where sugarcane is grown, plants are rarely subjected to water stress; consequently, it has been assumed that RSD is likely to cause little yield loss in Florida. However, RSD might be taking a small but substantial toll that goes unnoticed because of the otherwise favorable growing conditions for sugarcane. Experimental yield trials have shown that RSD causes significant yield losses in some clones in Florida (5,23); however, virtually nothing is known about the effects of RSD on commercial sugarcane production.

Because RSD cannot always be diagnosed accurately in the field, laboratory techniques to determine the presence or absence of the causal bacterium in xylem sap extracts from the host have been developed. Light (7) and electron (21) microscopy alone or combined with specific serological techniques, including fluorescent-antibody staining (11) and serologically specific electron microscopy (1), have been used for RSD diagnosis. Fluorescent-antibody staining is 10–100 times more sensitive than phase-contrast microscopy (11) but is more expensive. Culture media have recently been developed for the RSD bacterium, making it possible to base diagnosis on isolation and identification in culture (2,6). An enzyme-linked immunosorbent assay was investigated for diagnosis of RSD, but problems with nonspecificity occurred (9). The relative accuracies of any of these diagnostic techniques for RSD have not been compared under practical conditions.

The purpose of this research was to examine diagnostic procedures for RSD in sugarcane and use these procedures to ascertain the incidence of RSD in commercial plantings of sugarcane in Florida. This was viewed as a first step in determining the importance of RSD in Florida. Also, effective diagnosis is essential to other areas of RSD research.

MATERIALS AND METHODS

Plants of sugarcane clone CP 53-1 were grown from cuttings that had been inoculated with crude juice from RSD-infected sugarcane (10). Plants were grown in plastic pots in a greenhouse for 6–8 mo before use in preliminary comparisons of diagnostic techniques. Further tests were made with sugarcane samples from experimental or commercial fields. Only the first crop of each field planting was sampled.

Four experimental fields were established on land provided by sugarcane growers and maintained by the growers under conditions for commercial production. Planting material for the experimental fields came from a nursery of RSD-inoculated and uninoculated clones. Stalks of clones CP 59-22, CP 65-357, CP 70-1133, CP 72-1210, and CP 72-2086 were first heat-treated (23) to kill any naturally occurring RSD bacteria before inoculating (2) a portion of single-bud cuttings from the stalks with the F-1 strain of the RSD bacterium isolated from a plant of CP 53-1 in the earlier experiment. In January 1984, single-stalk samples were taken from five inoculated plants and one uninoculated plant of each of the five clones in each of the four experimental fields.

Commercial fields were sampled to compare diagnostic techniques and to determine the incidence of RSD in commercial sugarcane. Single-stalk samples were collected from three plants in each commercial sugarcane field on 1 September 1982 and again on 26 October 1982. Fields of clones CL 54-378, CL 59-1052, CP 65-357, CP 70-1133, and CP 72-1210 were sampled from each of four growers.

Because the RSD bacterium is xylem-inhabiting (4,7,22), xylem sap extracts were used in all diagnostic tests. Sap was extracted by centrifugation of stalk internodes (2). Before extraction, each stalk was washed with soap and running water, using a scouring pad to remove surface grime. If isolation in culture was to be attempted, the lower three or four internodes of each clean stalk were surface-sterilized by submersion in 70%

Florida Agricultural Experiment Stations Journal Series 5362.

Accepted for publication 21 April 1984.

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ethanol for 1–2 min, then dipped in 90% ethanol and flamed. Metal instruments were similarly sterilized between samples. A single, undamaged internode was selected from each stalk, and a portion (3–4 cm) was excised with pruning shears. This sample was placed with the apical end down in a sterile, 50-ml, conical-bottom, centrifuge tube. If an internode sample was too large to fit easily in a centrifuge tube, a longitudinal core removed with a 2.5-cm-diameter cork borer was used. Samples were centrifuged at 6,000 rpm for 5 min, and the sap extract was saved. Extracts were sometimes stored in sealed tubes at −20 C for a few days before examination by diagnostic techniques not requiring viable bacteria.

For isolation of the RSD bacterium in culture, fresh sap extracts were immediately placed on SC agar by the streak-plate or dilution-plate techniques (2). For phase-contrast examination, 5 μl of sap was placed on a microscope slide, covered with a coverslip (18 × 18 mm) and examined at ×1,000. Taxoplasmosis slides (Bellco Glass Inc., Vineland, NJ) with eight 6-mm wells were used for ordinary fluorescent-antibody tests by indirect or direct staining (16). A 5-μl sample of sap was applied evenly to each well then air-dried, and heat-fixed. Indirect stains were performed using antiserum provided by A. G. Gillaspie as described previously (3,11). Direct stains were performed using slides coated with air-dried gelatin (1% solution).

A new direct immunofluorescent staining technique being developed for both detection and microscopic enumeration (M. J. Davis, *unpublished*) of the RSD bacterium was used as only a diagnostic technique in this study. Acridine orange direct-count (AODC) methods (14) and direct fluorescent-antibody staining methods (16) were adapted for this diagnostic technique. The AODC technique uses acridine orange, a DNA-binding fluorochrome, for nonspecific staining of aquatic bacteria and involves microscopic enumeration of these bacteria after they have been collected on the surface of membrane filters.

In our technique, fluorescein isothiocyanate (FITC), conjugated to immunoglobulin G (IgG) with specificity for the RSD bacterium, was used instead of acridine orange and the AODC methods were adapted for sampling small volumes of sugarcane sap extracts. Stains were made by gently mixing 0.05–0.10 ml of sap extract with an equal portion of dilute FITC-IgG conjugate and incubating the mixture for 30 min in the dark at room temperature. The mixture was then diluted with 2–3 ml of 0.01 M phosphate-buffered saline (0.85%) (PBS), pH 7.2, mixed vigorously, and filtered to collect any stained bacteria in the sample. Polycarbonate membrane filters (Nucleopore Corp., Livermore, CA) 13 mm in diameter with 0.2-μm pores were used (12). The filters were stained overnight with an alcoholic solution of Sudan black B (0.007%) to provide a suitable background (26), then briefly rinsed with deionized water and air-dried. A Swinnex filter unit (Millipore Corp., Bedford, MA) was used to support the polycarbonate filter and a 0.45-μm pore-size nitrocellulose filter (Gelman, Ann Arbor, MI) was placed under the polycarbonate filter to prevent aggregation of cells and debris over pores in the membrane support. A 5-ml syringe was used to provide positive pressure for filtration. After filtration, the polycarbonate filter, while still moist, was carefully transferred to a microscope slide and a small drop of low-fluorescence immersion oil (Olympus Corp. of America, Lake Success, NY) was used to gently mount a coverslip on the filter. The surface of the filter was then examined for stained bacteria using epifluorescent microscopy at ×1,200.

The FITC-IgG conjugate for direct fluorescent-antibody staining was prepared with rabbit antiserum produced against whole cells of the L1A strain of the RSD bacterium from Louisiana. Cells of the bacterium were removed from the surface of heavily inoculated SC agar (2,6) after 10–14 days of incubation at 28 C. The cells were washed twice with PBS by centrifugation and resuspended in PBS at about 5×10^{10} cells per milliliter. The suspension was stored frozen at −20 C until used for immunization.

Inject antigen was prepared immediately before use by emulsifying 1.2 ml of cell suspension with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, MI). During 1 day, multiple injections of the inject antigen were used to immunize a New Zealand white rabbit. Twenty equally spaced intradermal injections in the sides and back consisting of 0.05–0.10 ml of emulsified antigen per site and intramuscular injections of 0.25–0.50 ml of emulsified antigen placed in each hip were given. Blood was collected 22 days later, and antiserum with an agglutination titer of 1,024–2,048 against 1×10^9 cells per milliliter of the L1A strain was obtained. The IgG from the antiserum was purified by affinity chromatography using Protein A on Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ) (17).

For conjugation with FITC, 6.4 ml of purified IgG containing about 2.5 mg/ml of protein was dialyzed for 12 hr at 4 C against three changes, 1,000 ml each, of 0.05 M borate buffer, pH 9.3, and once against 0.3 M NaCl in borate buffer (1,000 ml) for 4 hr. The IgG was conjugated to FITC by subsequent dialysis against 0.125% FITC-Celite (Sigma Chemical Company, St. Louis, MO) in borate buffer for 6 hr at room temperature. The conjugate was separated from unbound FITC on a Sephadex G-25 (Pharmacia) column (18) and eluted in PBS containing 0.02% sodium azide. The conjugate had an FITC-to-protein absorbance ratio, A_{490}/A_{280}, of 0.9 and a dilution titer of 320 against the L1A strain in direct fluorescent-antibody tests on taxoplasmosis slides. The conjugate was stored at 4 C. Immediately before use, the conjugate was diluted 40-fold with PBS and filtered through a 0.2-μm pore-size Acrodisc membrane filter apparatus (Gelman).

RESULTS AND DISCUSSION

Sensitivity, specificity, and reliability are primary factors determining the accuracy of all diagnostic techniques for plant diseases including RSD. The sensitivity of each diagnostic technique employed in this study was limited by the volume of sap actually sampled (Table 1). Sensitivity can also be affected by bacterial viability when diagnosis involves isolation in culture. Sugarcane clone CP 53-1 is very susceptible to RSD and supports the development of relatively high populations of the RSD bacterium (8). When we examined sap extracts of CP 53-1, all 45 test plants were determined to be infected by the indirect fluorescent-antibody staining test. Bacteria resembling the RSD bacterium were seen in 40 of these same 45 extracts when phase-contrast microscopy was used. Attempts to isolate the RSD bacterium in culture from the extracts resulted in 38 and 36 successful isolations, respectively, when the dilution-plate and streak-plate techniques were used. Populations of the RSD bacterium in the extracts averaged 7.5×10^7 cfu/ml and ranged from 9×10^5 to 6×10^8 cfu/ml. Both overgrowth with faster-growing bacterial contaminants and growth inhibition apparently caused by diffusable products of some but not all of these contaminants adversely affected isolation of the slow-growing RSD bacterium. On dilution plates, contaminants were less frequent at high dilutions, and apparently

Table 1. Actual volume of each sample of xylem sap extract examined for the presence of the ratoon stunting disease bacterium in different diagnostic techniques

Diagnostic technique	Total volume (ml) examined/sample
Isolation in culture	
Dilution-plate method[a]	3.0×10^{-4}
Streak-plate method[b]	1.0×10^{-3}
Phase-contrast microscopy[c]	6.3×10^{-6}
Fluorescent antibody using:	
Taxoplasmosis slides[c]	6.3×10^{-5}
Membrane filters[c]	4.6×10^{-4}

[a] A 30-μl sample of a 1×10^{-2} dilution, which was the lowest dilution used, was examined.
[b] An inoculation loop with about a 1-μl sample capacity was used.
[c] Fifteen microscopic fields were examined per sample.

pure cultures of the RSD bacterium were often obtained.

Improved media formulations, possibly selective for the RSD bacterium, or better surface-sterilization procedures for the plant material might improve the efficiency of the isolation techniques. Failure to detect the RSD bacterium in some of the test plants by the phase-contrast technique may have resulted because of the small sap volume actually examined and the small size and Brownian movement of the RSD bacterium, which necessitated examination of different focal planes while attempting to find the bacteria.

When the RSD bacteria were concentrated on the surface of membrane filters rather than fixed to taxoplasmosis slides, an estimated sevenfold increase in the number of fluorescent-antibody-stained bacteria in each microscope field was predicted by comparing relative volumes of sap sampled (Table 1). In actual tests using five independent, twofold, serial dilutions of a suspension in PBS of the F-1 strain from culture, an average of 1.1 cells in 75 microscope fields (15 fields per dilution in each of the five dilution series) was observed on taxoplasmosis slides (direct staining method). By comparison, an average of 0.9 cells in 75 fields was observed on membranes at an eightfold greater dilution. These results agreed favorably with the predicted relative sensitivities of the two techniques.

In tests conducted with samples collected from experimental fields of healthy and ratoon stunting-diseased sugarcane, FASM was more effective for detecting infections with the RSD bacterium than direct fluorescent-antibody staining on slides (FASS) and phase-contrast microscopy. When 100 plants in the ratoon stunting-diseased fields were examined, the RSD bacterium was detected in 70% by FASM, 57% by FASS, and 44% by phase-contrast microscopy. Table 2 shows the observed frequencies of possible combinations of results from the three techniques. There was 69% complete agreement of results of the three techniques. Results of the phase-contrast technique were mostly (87%) negative when they disagreed with those of one or both of the fluorescent-antibody techniques, indicating that phase-contrast microscopy was the least sensitive of the three techniques. There was 85% agreement between the results of the two fluorescent-antibody techniques, and the FASM results were positive in 14 of 15 instances when these results disagreed. FASM was either more sensitive than FASS or false-positive results were obtained. However, false-positive results from any of the three techniques were infrequent at most, because examination of 20 healthy plants grown in the same fields resulted in detection of the RSD bacterium in only one plant and then just by the FASM technique.

Of the 20 ratoon stunting-diseased plants of each clone that were examined in the experimental fields, 20 of CP 59-22, 18 of CP 72-1210, 14 of CP 65-357, 9 of CP 72-2086, and 9 of CP 70-1133 were determined by FASM to be infected with the RSD bacterium. Because the clones had been inoculated on the same day and with portions of the same inoculum, variations in susceptibility to infection of the clones or in their inherent ability to support detectable population levels of the bacterium once infected were probably responsible for the differences in frequency of detection.

Further work is needed to determine the actual sensitivities of diagnostic techniques in terms of the probability of detecting low populations of the RSD bacterium. Also, more information is needed on the population dynamics of the

Table 2. Observed frequencies for possible combinations of results of diagnoses of ratoon stunting disease of sugarcane from experimental fields using fluorescent-antibody staining on membranes (FASM), fluorescent-antibody staining on slides (FASS), and phase-contrast microscopy

Possible combination of results[a]			
FASM	FASS	Phase-contrast microscopy	Observed frequency[b]
+[a]	+	+	40
+	+	0	16
+	0	+	4
+	0	0	10
0	+	+	0
0	+	0	1
0	0	+	0
0	0	0	29

[a] + = Positive diagnoses, 0 = negative diagnoses.
[b] Given as the number of times the combination of results was obtained out of 100 examined plants.

Table 3. Observed frequencies for possible combinations of results of diagnoses of ratoon stunting disease of sugarcane from commercial fields using phase-contrast microscopy, isolation in culture, and fluorescent antibody staining on membranes (FASM)

Possible combination of results[a]			
FASM	Phase-contrast microscopy	Isolation in culture	Observed frequency[b]
+[a]	+	+	52
+	+	0	16
+	0	+	13
+	0	0	7
0	+	+	0
0	+	0	1
0	0	+	0
0	0	0	22

[a] + = Positive diagnoses, 0 = negative diagnoses.
[b] Given as the number of times the combination of results was obtained out of 111 examined plants.

Table 4. Incidence of the ratoon stunting disease bacterium in commercially grown sugarcane in Florida as determined by phase-contrast microscopy, isolation in culture by the dilution-plate technique on SC medium, and direct fluorescent-antibody stains on membrane filters

	Percent infected plants as determined by:								
	Phase-contrast microscopy			Culture			Fluorescent-antibody stain		
Clone[a]	1 September[b]	26 October	Total	1 September	26 October	Total	1 September	26 October	Total
CL 54-378	54.5(1)[c]	100.0	78.3	75.0	90.9(1)	82.6	90.9(1)	100.0	95.7
CL 59-1052	33.3	100.0	66.7	66.7	83.3	75.0	91.7	100.0	94.8
CP 65-357	44.4(3)	75.0	61.9	66.7	75.0	70.8	55.6(3)	91.7	76.2
CP 70-1133	30.0(2)	58.3	45.4	25.0	40.0(2)	36.8	60.0(2)	83.3	72.7
CP 72-1210	41.7	50.0	45.8	50.0	41.7	45.8	50.0	60.7	58.3
Total	40.7	76.7	59.6	56.7	66.7	62.1	70.4	88.3	79.8

[a] Twelve plants of each clone were sampled on each date. Three plants of each clone were sampled from each of four growers' fields.
[b] Samples were collected in 1982 from the first crop after planting.
[c] Numbers of plants for which no data were obtained because of insufficient sap or contamination of cultures are contained within parentheses.

RSD bacterium in sugarcane so that diagnoses can be performed when populations are at appropriate levels.

Phase-contrast microscopy, isolation by the dilution-plate technique, and FASM were used for diagnosis of RSD in commercially grown sugarcane. A total of 120 plants were sampled and 95 plants (79%) were infected with the RSD bacterium as determined by at least one of the diagnostic techniques. Samples from 111 of the plants were examined by all three techniques, and the observed frequencies of possible combinations of results are given in Table 3. Results of the FASM technique agreed with results of at least one other technique for 94% of the samples, whereas results of the culture and phase-contrast techniques agreed for 87 and 86% of the samples, respectively. All FASM results were positive when they disagreed with those of the other two techniques. Conversely, all but one of the results were negative for the phase-contrast or isolation tests when their results disagreed with those of both other techniques. Thus, it again appears that the FASM technique was the most accurate of the techniques.

Incidence of RSD in commercially grown sugarcane in Florida appeared to be very high (Table 4), especially because only plant crops were examined. Estimates of incidence varied with the sampling date and the clone sampled as well as with the diagnostic technique employed. The RSD bacterium was consistently detected in fewer samples on 1 September 1982 than on 26 October 1982 (Table 3), but populations of the bacterium apparently declined between the two sampling dates (Table 5).

Very little is known about the population dynamics of the RSD bacterium in sugarcane, but perhaps the bacteria in older stalk tissue become entrapped in a matrix material such as has been reported (15,24,25) and cannot be easily extracted by centrifugation. Significantly different populations of the RSD bacterium were found in different clones of sugarcane (Table 5). On both sampling dates, CP 72-1210, CL 54-378, and CL 59-1052 had relatively high populations, whereas CP 70-1133 consistently had the lowest populations. Populations in CP 65-357 dropped from a relatively high level to a relatively low level between sampling dates. Interestingly, the relative populations (Table 5) and incidences (Table 4) of the RSD bacterium in different clones ranked much the same, except for CP 72-1210, which had a low incidence of RSD but a high population of the RSD bacterium in infected plants.

Table 5. Population of the ratoon stunting disease bacterium in fibrovascular sap extracts from field collected sugarcane clones grown in Florida

Date	Clone	No. of positive isolations[y]	Average colony count/ml[z]
1 September 1982	CP 72-1210	6	3.3×10^7 a
	CL 54-378	9	3.1×10^7 a
	CP 65-357	8	2.3×10^7 a
	CL 59-1052	8	3.2×10^7 ab
	CP 70-1133	3	6.3×10^6 b
26 October 1982	CP 72-1210	5	8.0×10^6 a
	CL 54-378	10	8.4×10^6 ab
	CL 59-1052	10	7.5×10^6 ab
	CP 70-1133	4	1.2×10^6 bc
	CP 65-357	9	2.1×10^6 c

[y] Twelve plants of each clone were sampled on each date. Three plants of each clone were sampled in each of four growers' fields.

[z] Only counts from plants from which positive isolations were obtained were used to calculate the averages. Average counts followed by the same letter for the same date were not significantly different (Waller-Duncan k-ratio t test; $k = 100$).

ACKNOWLEDGMENTS

This research was supported in part by grants from the USDA Special Research Grants Program (Grant 83-CRSR-2-2138) and the USDA Competitive Research Grants Office (Grant 80-CRCR-1-0532). Excellent technical assistance was provided by S. J. Florenz and B. A. DesJardin.

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