Ratoon Stunting Disease of Sugarcane: Serology

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ABSTRACT

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Antisera were developed against the bacterium associated with the ratoon stunting disease (RSD) of sugarcane. The bacterium was selectively concentrated from the juice of ratoon stunting-diseased (RS-diseased) sugarcane or sudangrass hybrid. The antisera caused clumping of the bacterium and removed the infectivity from juice prepared from RS-diseased sugarcane or sudangrass hybrid. No serological relationship could be shown between the RSD-associated bacterium and *Corynebacterium flaccumfaciens*, *C. michiganense*, or *C. fascians* by the microagglutination test. The microagglutination test was not applicable for the diagnosis of RSD in raw sugarcane juice from diseased plants because of the low number of bacterial cells per milliliter. The bacteria were not degraded by sonication, pyrrolidine, or enzyme treatments.

As in other vegetatively propagated crops, disease agents in commercial sugarcane (*Saccharum* hybrid) are passed from one vegetative generation to the next. The ratoon stunting disease (RSD) is among the most serious diseases of sugarcane and causes large losses of productivity in plants which show no obvious external symptoms. The lack of a rapid diagnostic technique applicable to mass screening has hindered progress in the control of the disease.

There are two types of commonly used diagnostic tests. One test depends on the evaluation of internal stalk symptoms which may require from 2 to 26 wk to develop (6, 10, 11, 12). The other test involves establishing the presence of a characteristic bacterium associated with diseased plants. This bacterium is visible under high magnification by phase-contrast microscopy (3) or by electron microscopy (14). Although identification by the latter methods requires little time, the technology involved severely limits the number of samples that can be examined.

Serological techniques have been used widely to screen propagating material for the presence of viruses in several crops, and the successful application of a serological method for RSD would simplify diagnosis greatly. Numerous attempts in the past to obtain an antiserum to the RSD agent with infected sugarcane as a source of antigen have failed (13, and Gillaspie, *unpublished*).

The causal bacterium has not been cultured. Therefore, attempts were made to obtain an antiserum to cells of the bacteria from infected plants. It seemed desirable to use an alternate host of the pathogen in addition to commercial sugarcane. Juice from diseased plants of the sorghum-sudangrass hybrid NB 280 S contained a high concentration of bacteria (15) and thus was a desirable source of antigen for immunization studies.

The bacterium associated with RSD has been characterized as a coryneform type based on the presence of septa (15). The *Corynebacterium* species recently have been divided into five serological groups. Only *C. fascians* (Tilford) Dowson, *C. flaccumfaciens* (Hedges) Dows., and *C. michiganense* (Smith) Jensen, which represent three of these serogroups (8), were available for testing.

This paper reports: (i) the preparation of antisera against the bacterium concentrated from ratoon stunting-diseased (RS-diseased) plants, (ii) the use of these antisera to study possible serological relationships between the RSD-associated bacterium and other organisms, and (iii) attempts to use the antisera for diagnostic purposes.

MATERIALS AND METHODS

The RSD-associated bacteria were concentrated selectively by differential centrifugation and used for injection and serological tests. The schedule which yielded the highest concentration of bacteria in relation to the amount of host material is described here. Juice was milled from RS-diseased sudangrass hybrid NB 280 S plants which had set seed. A mixture of juice (40 ml), distilled water (60 ml), and acid-washed Celite (5 g) was filtered through Whatman No. 4 paper. The filtrate was centrifuged (12,000 *g* for 1 hr), the pellets were resuspended in buffered physiological saline (PBS) (0.01 M phosphate buffer, pH 7, containing 0.85% NaCl), centrifuged again (1,500 *g* for 10 min) and the supernatant fluid was diluted with water to a final volume of 45 ml. The diluted sample was layered onto an equal volume of 30% sucrose in water before centrifugation (52,000 *g* for 20 min). The pellets were resuspended in a total volume of 3.5 ml PBS, then centrifuged at 1,500 *g* for 10 min. The

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final supernatant fluid was slightly green and contained about 1.5×10^8 bacteria/ml as determined by phase-contrast microscopy. The supernatant fluid was frozen in 0.5-ml aliquots and used for injection of rabbit A. The antigen prepared for rabbit A contained a greater number of the RSD-associated bacterium than antigens prepared for injection of rabbits B, C, or D.

Rabbit B was injected with bacteria prepared from juice (10 to 30 ml) of diseased sudangrass which had been filtered through Celite and centrifuged at 12,000 *g* for 1 hr. The pellets were resuspended in 1 ml of PBS. Samples were injected immediately without prior freezing. Rabbit C was injected with samples concentrated from 200 ml of diseased sudangrass juice repeating the procedure for rabbit B antigen except that the pellets were resuspended in 3 ml of PBS. The sample was frozen in 0.5 ml aliquots and was diluted 1/7 before injection.

Antigen for rabbit D was prepared from RS-diseased sugarcane, CP 44-101, by the method of Gillaspie et al. (4). Juice (700 ml) was stirred with 78 ml of a solution containing 0.33 M KH_2PO_4, 0.67 M K_2HPO_4, and 0.1 M Na_2SO_3. The sample was centrifuged at 3,000 *g* for 30 min and the pellets were resuspended in 0.01 M Na_2SO_3. This was followed by centrifugation at 1,000 *g* for 30 min and then at 16,000 *g* for 1 hr. Pellets were resuspended in 11 ml of PBS and frozen in 1-ml aliquots. Aliquots were centrifuged at 1,500 *g* for 10 min before injection.

To produce the antisera, rabbits were injected repeatedly with bacterial samples over a 3- to 5-mo period (rabbit A – 3 mo, rabbit B – 4 mo, and rabbits C and D – 5 mo). Intramuscular injections of samples emulsified with Freund's complete adjuvant were followed by a series of intravenous injections of samples in PBS. Rabbits B and D were injected intramuscularly (0.5 to 1.5 ml emulsified preparation per injection) and intravenously (0.3- to 1.0 ml of preparation per injection) alternately and rabbits A and C were injected with three to five intramuscular injections (0.5 to 0.9 ml of emulsified preparation per injection) at 1-wk intervals followed by intravenous injections (0.3 to 0.5 ml of preparation per injection) at 4- to 10-day intervals. Bleedings were made at 4 to 8 days after some of the intravenous injections. Antisera were cross-absorbed at 37 C for 1 hr with pellets from healthy sudangrass hybrid juice. The pellets were obtained by centrifuging the juice at 12,000 *g* for 1 hr. Cross-absorption was repeated until all antibodies to host materials were removed.

Cultures of *Corynebacterium flaccumfaciens* (ICPBCF6), *C. michiganense* (ICPBCM9), and *C. fascians* (ICPBCF19), were provided by M. P. Starr from the International Collection of Phytopathogenic Bacteria at Davis, California. *Corynebacterium flaccumfaciens* and *C. michiganense* were each grown in 523 broth (7) at 27 C to produce antigens for immunization. After 5 days, the broth (500 ml) was centrifuged (12,000 *g* for 1 hr) and the pellets were resuspended in PBS. The samples were diluted until they were only slightly turbid and then frozen until needed for injection. Rabbits were injected over a 6-wk period starting with three intramuscular injections of bacterial suspension emulsified with Freund's complete adjuvant (0.4 to 0.7 ml of emulsified preparation/injection) at 1-wk intervals followed by a series of intravenous injections (0.5 ml of preparation/injection) at 3 to 7-day intervals. The rabbits were bled 12 and 27 days after the last intramuscular injection.

Antiserum for microagglutination tests was diluted in PBS and antigens were prepared by methods similar to those used to prepare antigen for rabbit A. Test plates were incubated at 28 C and were read at 4 hr and again after being kept overnight at 4 C.

A neutralization of infectivity test was performed with the antisera produced against the RSD-associated bacterium (RSD-antisera) and Celite-filtered juice from RS-diseased plants of sudangrass hybrid and of sugarcane cultivar Coimbatore. Juice samples (4.9 ml) were incubated at 37 C for 2 hr with 0.4 ml of either RSD-antiserum (rabbits A and D), a 1:4 dilution of RSD-antiserum, or normal serum. All 10 samples were centrifuged at 1,500 *g* for 10 min and the supernatant fluids were carefully removed with pipettes. An additional 0.1 ml of nondiluted serum was added to each sample and the samples were incubated for 1 hr at 37 C. The samples were centrifuged at low speed as above. Again 0.1 ml of serum was added and the samples were incubated and centrifuged. Tenfold dilutions of the samples were made to 10^{-3} and each was assayed on cuttings of CP 44-101 (6).

RESULTS

Comparison of RSD-antisera.—The antisera were evaluated with a single preparation of the RSD-associated bacterium from sudangrass (Table 1). This preparation contained about 6.5×10^9 bacteria/ml. Rabbit D was injected with a preparation from RS-diseased sugarcane containing a high ratio of host material to the bacteria. The titer in rabbit D to the RSD-associated bacterium increased slowly, but reached 1,024 after 137 days.

Rabbit B had been injected with sudangrass samples which contained large amounts of bacteria and host contaminants. The titer reached 512 at 108 days, but the rabbit died of anaphylactic shock after 121 days, and this method was discontinued.

Rabbit C had been injected with sudangrass samples similar to those of rabbit B, but diluted 1/7. The titer of RSD-associated bacterium at 49 days was 640 and it remained stable through 130 days.

Rabbit A had been injected with the cleanest sudangrass samples. The titer reached 640 after 27 days and was 512 after 71 days. The final bleeding had a titer of only 320, but this bleeding was made 15 days after the last injection. The rabbit A antiserum was easily cross-absorbed, but the antisera from rabbits B, C, and D required two to three incubations with pellets from juice of healthy plants to achieve complete cross-absorption. Clumping of the RSD-associated bacterium was observed by phase-contrast microscopy in samples reacted with the cross-absorbed antisera but no clumping was observed with the normal sera.

Serological tests.—Microagglutination tests were performed with antiserum prepared against the RSD-associated bacterium, and with antisera prepared against *C. flaccumfaciens* and *C. michiganense*. The antisera were reacted with antigens of *C. flaccumfaciens*, *C.*

michiganense, *C. fascians*, and the RSD-associated bacterium from sudangrass hybrid. The homologous titers were 2,048 for *C. flaccumfaciens* antiserum with antigen containing about 24.6×10^9 bacteria/ml, 128 for *C. michiganense* antiserum with antigen containing about 2.5×10^9 bacteria/ml, and 640 for RSD-antiserum (rabbit A) with antigen containing about 6.5×10^9 bacteria/ml. All homologous and heterologous combinations were tested, but only homologous reactions were positive. *Corynebacterium fascians* antigen contained about 1.5×10^9 bacteria/ml, but it reacted with none of the three antisera. A nonspecific reaction was observed between *C. flaccumfaciens* antigen and RSD-antiserum or normal serum. The lack of heterologous reactions among the three species of *Corynebacterium* agrees with a previous report (8).

The antisera prepared against antigen from RS-diseased sudangrass did react with the concentrated bacterial preparations from RS-diseased sugarcane cultivars L 62-96, Coimbatore, or CP 44-101 when these were prepared by a method similar to that for rabbit A antigen. Reactions with antigen in nondiluted sugarcane juice were unsatisfactory with the microagglutination test. Even with the highest titer antiserum, a concentration of about 2.5 to 5×10^7 bacteria/ml was necessary for a reaction. Few infected sugarcane clones contain this number of bacteria.

In the neutralization of infectivity test, very few (<1/field) or no bacteria were observed by phase-contrast microscopy in the samples incubated with RSD-antisera. However, bacteria were present (10 per field in Coimbatore and too many to count in sudangrass hybrid) in samples incubated with normal serum. The cuttings inoculated with sudangrass hybrid extracts incubated with antiserum were not infected, but those inoculated with 10^{-1} and 10^{-3} dilutions of extract incubated with normal serum yielded 100% and 25% infected plants, respectively. The cuttings inoculated with the Coimbatore extracts incubated with antiserum yielded only one infected plant from each of three nondiluted extracts, but those inoculated with extracts incubated with normal serum gave results similar to those with extracts from infected sudangrass hybrid.

Lysis of the bacterium.—Concentrated samples of RSD-associated bacteria were treated unsuccessfully by several methods of degradation. The cell-lysing method of Marmur (9) using lysozyme and detergent was attempted. Also, sonication, pyrrolidine treatment, and freezing and thawing were tested. No significant amount of lysis was observed with the RSD-associated bacteria under phase-contrast microscopy. The lysozyme-SDS treatment did release DNA from the *Corynebacterium* species (Davis and Gillaspie, *unpublished*), but the cells were not completely lysed as judged by phase-contrast microscopy. Neither the three *Corynebacterium* species nor the RSD-associated bacterium produced precipitin lines in immunodiffusion tests following these treatments.

DISCUSSION

The procedure used to prepare the antigen for injection of rabbit A had several advantages. The bacteria-containing sample could be injected at a higher concentration than the others because there was less host material present, and there was, therefore, less danger of causing anaphylactic shock in the rabbit. A fairly high titer could be reached in a short time (27 days). A higher titer probably would have been achieved if the number of bacteria in the intravenous injections had been increased progressively.

Cross-absorbed antisera cause specific clumping of the RSD-associated bacterium and should be useful in determining whether bacteria isolated from RS-diseased plants onto culture media are the RSD-associated bacteria. The cross-absorbed RSD-antisera are specific to the extent that they do not react with the *Corynebacterium* antigens. The RSD-antisera react with the RSD causal agent, as shown in the neutralization of infectivity, and with the associated bacterium, but this is not proof that the associated bacterium is the causal agent.

The lack of serological reaction between RSD-antisera and the RSD-associated bacterium in nonconcentrated sugarcane juice limits the usefulness of the microagglutination method for diagnosis. The method could be used for screening samples if they are concentrated, but the centrifugation required would limit the number of samples. A technique based on electron microscope grids activated with the RSD-antiserum has been reported to be sensitive enough for diagnosis from nontreated juice (2), but this technique is not an inexpensive serodiagnostic method for RSD. Methods such as bentonite flocculation (1) may be sensitive enough

TABLE 1. Titers of antisera to the bacterium associated with ratoon stunting-diseased sugarcane and sudangrass hybrid

Rabbit[a]	Injections[b] IM	Injections[b] IV	Days after first injection	Titer[c]
A	3	1	27	640
	0	6	70	512
	0	2	92[d]	320
B	5	5	51	320
	2	4	108	512
C	4	5	49	640
	0	5	74	640
	0	6	118	512
	0	2	131	640
D	4	8	45	16
	1	4	109	512
	0	7	137	1,024

[a] Rabbits A, B, and C injected with bacterial preparations from RS-diseased sudangrass hybrid and rabbit D with preparations from RS-diseased sugarcane.

[b] Intramuscular (IM) and intravenous (IV) injections were given as shown prior to the adjacent bleeding and since the previous bleeding. Generally, the IM's came first and were followed by IV boosters.

[c] Titers were obtained with a single preparation of RSD-associated bacteria from sudangrass hybrid. The estimated concentration of the preparation was about 6.5×10^9 bacteria/ml.

[d] This bleeding was 15 days after the last injection; all others were at 4 to 8 days after injection.

to be useful in screening for resistance. Since varieties susceptible to RSD-injury contain higher numbers of the associated bacterium (5), a serological method may be useful for detection only of those varieties with high bacterial numbers.

The lack of reaction between the *Corynebacterium* species antigen and the RSD-antisera and between the *Corynebacterium* species antisera and the RSD-associated bacterial antigen is interesting since in situ and in vitro microscopy has shown the presence of coryneform organisms associated with RS-diseased plants (4, 14, 15). There are two other serogroups of *Corynebacterium* (8), however, and there are other organisms, such as the actinomycetes, that are coryneform. Representatives of these groups of organisms will be tested in the future. Determination of serological relationships could be useful in selection of media for the possible culture of the RSD-associated bacterium.

The failure to find a method for lysing the RSD-associated bacterium prevents the use of immunodiffusion tests for the present. The significance of the resistance to lysis of the RSD-associated bacterial cells in determining taxonomic relationship is unknown.

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