Infectivity Titrations of *Clavibacter xyli* subsp. *xyli* and Sugarcane Cultivars Differing in Susceptibility to Ratoon Stunting Disease

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ABSTRACT

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Single-bud cuttings from healthy stalks of eight sugarcane cultivars differing in susceptibility to ratoon stunting disease were inoculated with 10- or 1,000-fold serial dilutions of the F-1 strain of *Clavibacter xyli* subsp. *xyli*. Inoculum levels ranged from 10^1 to 10^7 cfu/ml. Sap from mature stalks of plants grown from cuttings was examined for *C. x.* subsp. *xyli* with phase-contrast microscopy and/or a fluorescent-antibody staining procedure. Probit analyses of quantal responses of sugarcane cultivars to *C. x.* subsp. *xyli* gave $\log_{10}$ ED_{50} values (dosage per milliliter required to achieve infection of 50% of the population) from 2.87 for cultivar CP 53-1 to 12.3 for H 60-6909. ED_{50} values were inversely correlated with yield-loss estimates for the cultivars.

Ratoon stunting disease (RSD) of sugarcane (interspecific hybrids of *Saccharum*) caused by the xylem-inhabiting, coryneform bacterium, *Clavibacter xyli* subsp. *xyli* (Davis et al) (3), is an insidious disease because it can substantially reduce yield without producing readily apparent symptoms (10). The lack of characteristic symptoms has made diagnosis of RSD and rapid assessment of disease severity in the field difficult (9). Therefore, the incidence and importance of the disease has been difficult to accurately assess. However, largely because of yield losses and the ubiquitous distribution of *C. x.* subsp. *xyli* throughout the sugarcane-growing areas of the world, RSD is considered the most important disease affecting commercial sugarcane production (6).

Development of resistant cultivars as a strategy for controlling RSD has been secondary to other control practices. Heat treatment of planting material and sanitation are the principal measures used to prevent reintroduction and/or spread of the pathogen (10). Several factors have hampered the exploitation of genetic resistance in sugarcane. These include the lack of a rapid means of assessing the severity of RSD by symptomatology, the inability for a long time to identify and isolate the causal organism, and the lack of practical methods for rapidly screening large numbers of cultivars for resistance to the disease. Screening for resistance has been achieved by yield-loss trials, which are both time-consuming and expensive. Consequently, such screening has not been routinely conducted in most breeding programs.

Although no cultivars of sugarcane are immune to infection by *C. x.* subsp. *xyli*, resistance to the disease has been recognized in some (11,12,15). Studies on the basis for this resistance indicate that innate structural features of the vascular systems of resistant cultivars may play an important role by restricting the spread of the pathogen in the xylem (11,12). Correlations have been found between rates of water movement (11) or reduction in water flow (14) through single-node cuttings of stalks and resistance of these cultivars to RSD. This resistance was associated with profuse branching of the metaxylem vessels at the nodes (11). Also, studies of the movement of india ink particles in the xylem (12) indicated that more resistant cultivars have fewer vessels that pass directly through nodes without terminating. Preliminary screening of sugarcane for resistance to RSD on the basis of vascular anatomy and hydraulic conductivity has been suggested (11,14).

Recent advances in RSD research, including isolation of the causal organism in axenic culture (2) and development of sensitive techniques for detection and enumeration of *C. x.* subsp. *xyli* populations in sap from infected plants (1), have made further studies of the nature of sugarcane-RSD interactions and development of additional screening techniques possible. Inoculum-dose experiments have proved useful for assessing resistance of plants to several bacterial plant pathogens (5) and may provide an alternative approach to evaluate sugarcane cultivars for resistance to RSD.

The purpose of this research was to evaluate the relative resistance or susceptibility of sugarcane cultivars to infection by different doses of *C. x.* subsp. *xyli*. Analyses of dose-response data were used to determine the median effective dose (ED_{50}) necessary for successful infection of each cultivar. The relationship between ED_{50} estimates and RSD severity as determined by yield-loss assessment was established.

MATERIALS AND METHODS

Plants of eight sugarcane cultivars, CP 53-1, CP 72-1210, CP 65-357, CP 63-588, CP 70-1133, CP 72-2086, CP 29-116, and H 60-6909, were used as sources of planting material for this study and were established from seed cane that had been

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heat-treated (13) to eliminate any naturally occurring RSD bacteria. The plants were maintained at the Research and Education Center, Ft. Lauderdale, and were judged to be free of RSD only after the pathogen was not found during periodic microscopic examinations of their sap.

Inoculum consisted of 7-day-old RSD broth (3) cultures of the F-1 strain (1) of *C. x.* subsp. *xyli* at the mid-log phase of growth. These broth cultures contained about 10^8 cells per milliliter as determined by a fluorescent-antibody (FA) staining procedure (1). For inoculum, serial 10- and 1,000-fold dilutions of the cultures were prepared in 0.01 M phosphate-buffered saline (0.85%), pH 7.2. The concentration of viable cells (cfu/ml) in the inoculum was determined by plating on SC medium (2). Colonies were counted after 2–3 wk of incubation at 29 C. Dilutions were prepared immediately before use.

Single-bud cuttings 4 cm long were excised from mature stalks of sugarcane plants with pruning shears. In experiment 1, cuttings of all eight of the aforementioned cultivars were inoculated with 10^7, 10^4, or 10^1 cfu/ml of *C. x.* subsp. *xyli*. In experiment 2, cuttings of cultivars CP 53-1, CP 72-1210, and CP 72-2086 were inoculated with a series of seven 10-fold dilutions of *C. x.* subsp. *xyli* cultures ranging from 10^1 to 10^7 cfu/ml. For each treatment, about 40 cuttings of each cultivar were inoculated by placing them in plastic bags and adding sufficient inoculum to totally immerse them. After 1 hr, excess inoculum was decanted, the bags were sealed, and the cuttings were stored overnight at room temperature. Inoculated cuttings were air-dried before planting in potting soil in flats. One-month-old plants were transferred to 9-cm-square plastic pots and, after 2 mo of additional growth, transplanted to a field at the USDA Sugarcane Field Station, Canal Point, FL, in July 1984. A randomized complete block design was used for each experiment with each cultivar and inoculum level replicated once in each of three blocks. Thirty plants were used for each treatment, thus each treatment replicate comprised 10 plants.

Assessment of infection. Because infection by *C. x.* subsp. *xyli* does not produce any overt external symptoms in sugarcane and because not all sugarcane cultivars develop reliable internal symptoms, plant infection was assessed by detecting the bacterium in sap extracts of stalk internodes. In January 1985, single-stalk samples were taken from inoculated plants by severing them at the soil line. Sap was collected from each stalk by centrifugation of the first undamaged basal internode by methods described previously (1). Ten-microliter quantities of sap from each sample were viewed by phase-contrast microscopy at ×1,200 and rated either positive or negative for *C. x.* subsp. *xyli*. Extracts rated negative were examined further by FA staining and epifluorescence microscopy at ×1,200 (1). In both procedures, plants were rated positive for *C. x.* subsp. *xyli* infection if counts of the pathogen in sap samples contained one or more bacteria per field in 15 microscope fields.

The probit procedure (Statistical Analysis Systems Inc., Cary, NC) was used to analyze the quantal response data from each experiment. ED_{50} values were compared with yield-loss estimates of the cultivars. These estimates were obtained from replicated single-stool trials conducted, as described previously (4) during 1979–1984 at the USDA Sugarcane Field Station, Canal Point. Briefly, the trials consisted of a randomized block design with a paired comparison of a healthy and a diseased single-stool plant of each cultivar in each of 20–30 blocks. Only plant crops were examined. A compilation of the yield-loss values for each cultivar from three separate trials, expressed as percent mean reduction in weight, was used as the best estimate of the relative susceptibility of each cultivar to RSD.

An analysis of variance of the regression of yield-loss values for test cultivars on their corresponding ED_{50} values was performed with total sums of squares partitioned to estimate the effects of the linear and quadratic components of this relationship.

RESULTS

Infectivity titrations. The inoculum-dose responses for different sugarcane cultivars were variable (Table 1). Percent infection ranged from 100% for CP 53-1 (experiments 1 and 2) and CP 72-1210 (experiment 1) to 10% for CP 29-116 and H 60-6909 (experiment 1) for the highest inoculum level (10^7 cfu/ml) tested. In all cases, the numbers of infected plants of each cultivar progressively increased as inoculum levels increased. *C. x.* subsp. *xyli* was not detected in plants of cultivars CP 29-116 (experiment 1) and CP 72-2086 (experiment 2) after seed pieces were inoculated with 10^4 cfu/ml or lower concentrations. By comparison, the pathogen was detected in sap from CP 53-1 after inoculation with 100- and 1,000-fold greater dilutions of *C. x.* subsp. *xyli*.

Log-dose/probit-response relationships. The parameters describing the log-dose/probit-response relationships based on the quantal responses of the sugarcane cultivars to infection by *C. x.* subsp. *xyli* are presented in Table 2. Chi-square tests on the dose-response data for cultivars indicate no departure of the observed from the expected values. In all cases, the probit slope (regression coefficient) values for each infectivity titration was less than 2, indicating that *C. x.* subsp. *xyli* cells probably act independently (5) to establish progressive infections in sugarcane.

The $\log_{10}$ ED_{50} values and their 95% fiducial limits for each sugarcane cultivar are also presented in Table 2. The cultivars may tentatively be ranked in decreasing order of susceptibility to infection by *C. x.* subsp. *xyli* on the basis of these values as follows: CP 53-1, CP 72-1210, CP 63-357, CP 63-588, CP 70-1133, CP 72-2086, and H 60-6909. The cultivar CP 29-116 was also included in the first experiment; however, because only three inoculum dilutions were tested and no infected plants were obtained for two of the three dilutions, a probit regression line and ED_{50} value could not be calculated for this cultivar. Because CP 29-116 and H 60-6909 had very similar frequencies of infection at all inoculum doses, their ED_{50} values should be similar.

Relationship between yield loss and ED_{50}. A high inverse correlation ($r = -0.976$; null hypothesis: slope = 0, $P = 0.002$) was found between yield reduction caused by RSD and the corresponding ED_{50} values for the cultivars tested in experiment 1. An analysis of variance of the regression (Table 3) indicated that both the linear and quadratic components

Table 1. Incidence of ratoon stunting disease in mature plants of sugarcane cultivars propagated from single-bud cuttings inoculated with different doses of *Clavibacter xyli* subsp. *xyli* as detected by phase-contrast and/or immunofluorescence microscopy

Cultivar	Inoculum dose ($\log_{10}$ cfu/ml)						
	7	6	5	4	3	2	1
	Experiment 1						
CP 53-1	30/30[a]	···	···	26/30	···	···	1/30
CP 72-1210	30/30	···	···	10/30	···	···	0/30
CP 65-357	28/30	···	···	7/30	···	···	0/30
CP 63-588	27/30	···	···	7/30	···	···	0/30
CP 70-1133	23/30	···	···	7/30	···	···	0/30
CP 72-2086	7/30	···	···	4/30	···	···	0/30
H 60-6909	3/30	···	···	1/30	···	···	0/30
CP 29-116	3/30	···	···	0/30	···	···	0/30
	Experiment 2						
CP 53-1	30/30	30/30	28/30	21/30	2/30	1/28	0/30
CP 72-1210	26/30	22/30	20/30	13/30	4/30	0/30	0/30
CP 72-2086	9/30	6/30	2/29	0/30	0/30	0/30	0/30

[a] Number of plants infected with *C. x.* subsp. *xyli*/number of plants inoculated.

Table 2. Parameters of regression lines that describe the log-dose/probit-response relationships of *Clavibacter xyli* subsp. *xyli* and sugarcane cultivars differing in susceptibility to ratoon stunting disease

Cultivar	Probit analyses[a]				Yield loss[b]
	Number of iterations	Regression equation	$P >$ chi-sq.	Log_{10} ED_{50} (95% fiducial limits)	
			Experiment 1		
CP 53-1	2	$Y = 2.18 + 0.98X$	0.98	2.87 (2.31, 3.36)	20.53
CP 72-1210	3	$Y = 1.48 + 0.77X$	0.73	4.56 (3.99, 5.14)	10.88
CP 65-357	3	$Y = 1.24 + 0.75X$	0.83	4.99 (4.43, 5.56)	8.69
CP 63-588	3	$Y = 1.50 + 0.69X$	0.76	5.10 (4.51, 5.70)	13.64
CP 70-1133	4	$Y = 1.93 + 0.55X$	0.57	5.45 (4.79, 6.17)	5.70
CP 72-2086	5	$Y = 2.68 + 0.24X$	0.22	9.67 (7.48, 22.80)	0.00
H 60-6909	4	$Y = 2.09 + 0.24X$	0.63	12.30 (8.51, ···)	−2.92
			Experiment 2		
CP 53-1	4	$Y = -0.41 + 1.33X$	0.31	4.06 (3.82, 4.31)	···
CP 72-1210	4	$Y = 2.02 + 0.59X$	0.25	5.02 (4.64, 5.39)	···
CP 72-2086	5	$Y = 0.76 + 0.49X$	0.60	8.59 (7.60, 11.80)	···

[a] Probit analysis programs of the Statistical Analysis System Inc., Cary, NC.
[b] J. L. Dean and M. J. Davis (*unpublished*). Values for cultivars, expressed as percent mean reduction in weight (kg/stool) caused by RSD, are from replicated single-stool yield trials consisting of paired comparisons of healthy and diseased plants. Trials were conducted during 1979–1984 at the USDA Sugarcane Field Station, Canal Point, FL.

Table 3. Analysis of variance of the regression of yield-loss estimates of sugarcane cultivars with ratoon stunting disease from single-stool yield trials on median effective dose (ED_{50}) values for successful infection of these cultivars by *Clavibacter xyli* subsp. *xyli*

Source	df	SS	MS	*F*	$P > F$
Total	6	65.927	···	···	···
Yield loss					
Linear	1	55.399	55.399	70.87	0.001
Quadratic	1	7.401	7.401	9.47	0.037
Error	4	3.127	0.782	···	···

of this relationship were significant, with 88.2% of the total sums of squares explained by the linear and 11.8% by the quadratic components, respectively.

DISCUSSION

Infectivity titrations based on quantal responses (i.e., the presence or absence of the pathogen in sap from stalks) provided a convenient means to rapidly determine the reactions of sugarcane cultivars to different doses of *C. x.* subsp. *xyli*. Such an approach is now feasible because of the recent development of culture media for *C. x.* subsp. *xyli* (2,3) and improved methods for detecting the pathogen (1). Although infectivity titrations such as these have not been reported previously, differential responses of cultivars after inoculation with sap from infected plants have been reported (8,15), with infection frequencies ranging from 60–80% for susceptible cultivars (8) to no detectable infections in the resistant cultivar H 60-6909 (15).

In general, progressively smaller inoculum doses below the level required for 100% infection resulted in fewer plants becoming infected until no further infections were observed. Additionally, a range of responses to different inoculum doses was observed among the cultivars examined. The dose response of the sugarcane cultivars to *C. x.* subsp. *xyli*, based on their ED_{50} values, differed such that the cultivars could be ranked in order of their relative susceptibilities to infection. Similar rankings were obtained for the cultivars tested in both experiments even though their respective ED_{50} values differed between experiments. The discrepancies in ED_{50} values might possibly be attributed to differences in the number of inoculum levels used in each experiment coupled with the inherently low precision of infectivity titrations based on quantal responses (7).

The high inverse correlation between yield-loss estimates and ED_{50} values suggests that infectivity titrations can provide useful indications of the relative resistance or susceptibility of sugarcane cultivars to RSD. In addition, this relationship also suggests that the factors and/or mechanisms determining yield loss might be the same as those that govern infection.

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