Quantitative Variability of *Clavibacter xyli* subsp. *xyli* Populations in Sugarcane Cultivars Differing in Resistance to Ratoon Stunting Disease

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ABSTRACT

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Cells of *Clavibacter xyli* subsp. *xyli*, the bacterium that causes ratoon stunting disease, were extracted by centrifugation from a basal internode of sugarcane stalks and enumerated using a fluorescent-antibody direct-count procedure. When pathogen populations in 11 cultivars were examined on different dates or when grown at two different locations, the distributions of density estimates for individual populations were frequently not normally distributed about the arithmetic mean. Furthermore, the means and variances of the different populations were positively correlated, but the coefficient of variation decreased with greater densities. Generally, a quartic-root transformation normalized the data for individual populations and eliminated the relationship between means and variances, thus validating the use of parametric statistics. Pathogen densities were different among sugarcane cultivars but increased at approximately the same rate within each crop during the time period sampled. A significant correlation was observed between mean population density estimates for cultivars when crops or geographic locations were compared. When yield reduction in cultivars due to ratoon stunting disease was determined at one location, it was significantly correlated with corresponding estimates of pathogen density.

Additional key words: data transformation, *Saccharum*, xylem-inhabiting bacterium.

Ratoon stunting disease (RSD) of sugarcane (*Saccharum*, interspecific hybrids) occurs in most, if not all, sugarcane-producing areas of the world (22) and often causes significant yield reduction (11). RSD is caused by *Clavibacter xyli* subsp. *xyli* (3, 4), a small, xylem-inhabiting, coryneform bacterium first reported to be associated with RSD in 1973 (6,15,26). Stunting is usually the only overt symptom of RSD but is not characteristic of RSD alone; consequently, both diagnosis of this disease and assessment of cultivar susceptibility are difficult (26). Yield trials comparing healthy and diseased plantings of sugarcane cultivars are the only proven method for determining the effect of RSD (5,12). Such trials are economically impractical for large-scale screening for resistance in breeding programs. Thus, the relative susceptibility to RSD is known for only a few cultivars in commercial production today.

Gillaspie et al (7) found that the relative population density of *C. x.* subsp. *xyli* in extracts from different sugarcane cultivars was positively correlated with their degree of susceptibility to RSD and suggested that measuring pathogen populations might provide a means to screen for resistance. Bailey (1) subsequently confirmed this relationship and demonstrated that it also held when respective populations for various parts of sugarcane plants were compared. These studies also demonstrated that greater populations of the pathogen developed in stalks than in leaves and that these populations increased as the tissues matured.

The precision and sensitivity of methods used to enumerate cells of *C. x.* subsp. *xyli* are important factors when considering the development of a screening procedure for resistance to RSD based on population densities. Recently, a direct-count technique using fluorescent-labeled antibodies (FADCF) was developed to enumerate *C. x.* subsp. *xyli* cells collected on membrane filters from sap extracts (2). This technique was more sensitive and/or specific than other direct-count techniques tested and was more reliable and rapid than enumeration of colonies following dilution-plating on culture medium. The frequency distribution and quantitative variability among density estimates for populations of *C. x.* subsp. *xyli* in sugarcane are also important considerations. Recent studies on epiphytic (10) and rhizosphere (14) bacteria showed that the frequency distribution of density estimates of these organisms often approximated a lognormal distribution. Consequently, both arithmetic means of density estimates and estimates obtained from pooled samples overestimated the size of the different populations. Additionally, the variance and means of density estimates, at least for rhizosphere bacteria, were directly correlated in the arithmetic scale but not in the log scale. Thus, assumptions for the analysis of variance when using parametric statistics—namely those of a normal distribution of experimental errors and of independence of means and variances, which would have been violated by using the arithmetic data—were generally met following a logarithmic transformation.

The purpose of this study was to examine quantitatively the temporal distribution and variability among *C. x.* subsp. *xyli* populations in sugarcane cultivars differing in resistance to RSD. Data transformations to enhance both the validity of analyses of variance and estimation of the central tendency of individual populations are discussed.

MATERIALS AND METHODS

Inoculation and propagation of experimental plants. Stalks of sugarcane cultivars CP 43-47, CP 44-101, CP 53-1, CP 59-22, CP 63-588, CP 65-357, CP 70-1133, CP 72-1210, CP 72-2086, CP 74-2005, and H 60-6909 were hot water-treated at 51 C for 2 hr to eliminate natural infections by *C. x.* subsp. *xyli* (27). Single-node cuttings from treated stalks were then immediately inoculated (3) with the F1 strain (4) of the pathogen in June 1982. Plants were grown from both inoculated and noninoculated cuttings in a nursery. These plants were used as a source of seedcane in December 1982 to establish experimental field plots of infected and noninfected plants at Ft. Lauderdale, FL. Plants for field plots were propagated, as before, from single-node cuttings and, after initial growth in a screenhouse, were transplanted to the field in

March 1983. Also, cultivar CP 53-1, which had been inoculated with crude juice preparations from sugarcane with RSD (5) and was the original source of the F1 strain of *C. x.* subsp. *xyli*, was similarly propagated and planted in the field. This constituted another cultivar-inoculum combination.

A second experimental planting consisting of both infected and noninfected plants of each cultivar was established at Canal Point, FL, on 11 May 1984. Plants propagated from seedcane, which had been harvested from field plots at Ft. Lauderdale on 5 March 1984, were used.

In addition, following the last sampling of plants during the first season of growth (plant crop) at Ft. Lauderdale, any remaining stalks were cut back to allow development of a ratoon crop from the stubble (first-stubble crop).

Experimental design. Experimental fields were planted at both locations using a randomized complete block design. At Ft. Lauderdale, each plot contained a row of five plants (subplots) of a single treatment consisting of either infected or noninfected plants of a single cultivar, with each treatment replicated once in each of three blocks. Supplemental irrigation was provided by an overhead sprinkler system. The soil was an Entisol (Margate fine sand). All plants were periodically examined for the presence of *C. x.* subsp. *xyli*, as described below.

At Canal Point, plots were split into subplots containing a paired noninfected and infected plant of each cultivar in each of 29 blocks. Supplemental irrigation was provided by regulating the water table depth. The soil was a highly organic Histisol (Torry muck).

Sampling procedures. To limit the effects that spatial and temporal distributions of *C. x.* subsp. *xyli* in sugarcane plants might have on estimates of population size, only the third internode above ground of the oldest two stalks (plant crop) or single stalk (stubble crop) were sampled from each plant on each date. Samples were limited to the lower portion of stalks because populations of the pathogen are greater in these tissues than in either the upper portion of stalks or leaves (1). At Ft. Lauderdale, each RSD-infected plant of both the plant crop and first-stubble crop was sampled three times at 6-wk intervals. All stalks sampled during each sampling period were harvested within 7–10 days of each other. Thus, the mean sampling dates were 18 October 1983, 2 December 1983, and 22 January 1984 for the plant crop and 25 June, 21 August, and 22 October 1984 for the first-stubble crop. Thus, 1,620 stalks were sampled at Ft. Lauderdale, 1,080 in the plant crop (two stalks per plant on each of three sampling dates), and 540 in the first-stubble crop (one stalk per plant on each of three sampling dates). For each of the 11 cultivars at Canal Point, a stalk was taken from each plant of 15 pairs of inoculated and noninoculated plants on 12 December 1984 to determine whether the plants were infected and for pathogen density measurements. Thus, 165 stalks were sampled from the Canal Point planting.

Although population densities of *C. x.* subsp. *xyli* are reportedly greater in nodal tissues (1), internodes were sampled in this study. Preliminary experiments (*unpublished data*) indicated that population densities were uniform throughout internodes and that similar numbers of cells were present in sap samples regardless of the direction of sap extraction from these tissues during centrifugation. By comparison, bacterial populations extracted from nodes varied, depending on the orientation of these tissues during extraction. Population densities in sap from nodes consistently exceeded those from internodes only when sap was extracted against the direction of transpirational flow, and then only when nodal sections included tissues from immediately below leaf scars. Sap was extracted from internodes by centrifugation at 8,300 *g* for 10 min, as previously described (4,5). Extracts were stored at 4 C if examined on the day of extraction; otherwise, they were stored frozen at −20 C and usually examined within a week. Sap was extracted from stalks usually within 3 hr of harvest, except when stalks from Canal Point were transported to Ft. Lauderdale on the day of harvest and stored overnight at 4 C before extraction.

Measurement of pathogen populations. Populations of *C. x.* subsp. *xyli* were enumerated using a FADCF technique and following a previously described protocol (2). Briefly, equal volumes, usually 0.1 ml, of an individual or pooled sap sample and an appropriately diluted fluorescein isothiocyanate-conjugated rabbit immunoglobulin G, specific for *C. xyli*, were mixed and incubated at room temperature for 30 min. Stained bacterial cells in each sap sample were then collected on a membrane filter and observed using epifluorescence microscopy at 1,200×. The area within each microscope field used for counting bacteria was adjusted with an eyepiece reticle for each sample according to the number of bacterial cells observed. Ten to 20 cells were usually counted per field, and fields were selected at random. Cells were counted in 15 fields or in fields until approximately 150 cells were counted, whichever came first. The number of cells per milliliter of sap was then calculated. The minimum concentration for enumeration was set at one cell per entire microscope field, which was equal to approximately 4×10^4 cells per milliliter of sap. Cell concentrations below this level were treated as missing data for the purpose of statistical analyses.

Measurement of the effects of RSD on sugarcane yields. For the Canal Point planting, the effects of RSD on yields were determined following previously described procedures (5), except that a pure culture of the F1 strain of *C. x.* subsp. *xyli*, not crude juice extracts from diseased plants, was used for the inoculum. All stalks of each plant were harvested and weighed on 12 December 1984.

Statistical analyses. Computer programs of two statistical packages, SAS version 5 (Statistical Analysis Systems, SAS Institute, Inc., Cary, NC) and Statpro version 1.3 (Penton Software, Inc., New York, NY) were used for data analysis.

Relationships between the variance and mean of pathogen density estimates for populations of *C. x.* subsp. *xyli* were examined using the SAS Reg procedure for regression analysis and SAS GLM (General Linear Models) procedure for covariance analysis. To enhance the accuracy of analyses using parametric statistics, variance stabilizing transformations were investigated after variance-mean relationships were found. Taylor's power law (21) was used to suggest possible transformations. These transformations were then optimized to produce zero correlation between the variance and mean by conducting an iterative computer search using the SAS Corr procedure to obtain values for the Pearson's correlation coefficient. A variance-range test (9,25) was then used to select the most appropriate transformation following procedures described by Logan (13). For these and some subsequent analyses, the data were divided into 71 data sets, each consisting of all individual density estimates obtained for a cultivar at one location during a sampling period, and block effects were disregarded. These data sets were considered to represent individual populations of *C. x.* subsp. *xyli*.

To determine whether the frequency distribution of density estimates within the data sets was normal, both the SAS Univariate procedure and Statpro normality testing procedure were used. The Univariate procedure computes the Shapiro-Wilk (18) test statistic, W, which is the ratio of the best estimator of the variance (based on the square of a linear combination of the order statistics) to the usual corrected sum of squares estimator of the variance; the Statpro procedure computes the quantile-quantile (Q-Q) correlation statistic that measures the correspondence between the cumulative probability distribution of the raw data and the corresponding values of the standard normal distribution on a quantile-quantile basis. Probability levels less than 0.01 for either normality test statistic were considered to indicate nonnormality of *C. x.* subsp. *xyli* data. To further determine whether distributions of the raw and transformed data affected the mean as a measure of the central tendency of the individual populations, skewness of the data was measured by computing the g_1 statistic (20) using the SAS Univariate or Means procedures. Skewness (g_1) is a measure of the tendency of the deviations from the mean to be larger in one direction than in the other. When data are skewed (the tail of the distribution curve is longer) in a direction greater than the mean, the value of g_1 is positive; conversely, when data are skewed in a direction less than the mean, the value of g_1 is negative. Excessive skewness in either direction indicates that the mean is an inappropriate estimator of the central tendency of the data.

The quantitative variability of *C. x.* subsp. *xyli* populations with

respect to different effects was assessed with the Ft. Lauderdale data using the SAS GLM procedure to compute covariance analyses and the SAS Varcomp procedure to compute variance component analyses. Plot means for appropriately transformed density estimates and the number of days after 1 January of the current growing season were derived for the covariance analyses. Days after 1 January were selected to standardize the measurement of time for both crops at Ft. Lauderdale. Additivity of treatment effects with respect to pathogen density was assessed using Tukey's test (19) for a two-way classification; plot means of appropriately transformed density estimates were used, and cultivar and sampling date were treated as the main effects. Variance component analyses were made on the plant crop data at Ft. Lauderdale by mean sampling date and by using the individual transformed density estimates for each sap sample; cultivar and block (row) effects were treated as fixed.

The SAS Corr procedure for correlation analyses was used to compare mean population density estimates for nine cultivars grown at Ft. Lauderdale with those of the same cultivars grown at Canal Point and to compare mean population density estimates with the effects of RSD on yield of all 11 cultivars grown at Canal Point.

RESULTS

Measurement of population densities. Sampling dates were chosen in the latter portion of each growing season after preliminary surveys indicated that the pathogen could be detected in most cultivars. Throughout the study, the pathogen was not detected in 29 of the 180 plants (15 plants for each of 12 cultivar-inoculum combinations), which were the vegetative progeny of inoculated plants and subsequently referred to as inoculated plants, comprising the Ft. Lauderdale planting. These 29 plants were distributed among cultivars as follows: CP 53-1, 3; CP 59-22, 2; CP 65-357, 2; CP 74-2005, 4; CP 72-2086, 8; and H 60-6909, 10. Additionally, the pathogen was not detected in one plant of cultivars CP 59-22 and CP 65-357 and three plants of CP 72-2086 in the stubble crop after it had been consistently detected in these plants in the plant crop. Spread of the pathogen to noninoculated plants did not occur during the study.

Of the 1,620 sap samples from inoculated plants at Ft. Lauderdale, 1,344 were from plants that were determined to be infected after the plants had been sampled repeatedly throughout the study. Of the latter samples, enumeration data were not obtained from three of these samples for various reasons, and *C. x.* subsp. *xyli* cells were not observed in 23 of the remaining 1,341 samples (1.7%). These 23 samples were from eight of the cultivars. *C. x.* subsp. *xyli* cells were observed at concentrations below the minimum level set for enumeration (one cell per microscope field) in 155 of the 1,341 samples (11.6%), leaving 1,163 samples for which count data were obtained. The percentage of samples from infected plants with cell numbers below the minimum for enumeration was 13.5, 9.4, and 8.1% in the plant crop on 18 October, 2 December, and 22 January, and 16.6, 12.7, and 12.7% in the stubble crop on 25 June, 21 August, and 22 October, respectively. For infected plants of each cultivar, the total number of samples in which cells were not detected or the cell numbers were below the minimum set for enumeration was as follows: CP 43-47, 0 (0%); CP 44-101, 4 (3.0%); CP 59-22, 1 (0.8%); CP 53-1 (juice-inoculated), 3 (2.2%); CP 53-1, 5 (5.2%); CP 63-588, 6 (4.4%); CP 72-1210, 14 (10.4%); CP 65-357, 18 (15.8%); CP 70-1133, 20 (15.3%); CP 74-2005, 34 (32.4%); CP 72-2086, 31 (56.4%); and H 60-6909, 42 (93.3%). The number of samples for which count data were obtained for each cultivar was as follows: CP 43-47, 135; CP 44-101, 131; CP 59-22, 119; CP 53-1 (juice-inoculated), 132; CP 53-1, 91; CP 63-588, 129; CP 72-1210, 121; CP 65-357, 96; CP 70-1133, 111; CP 74-2005, 71; CP 72-2086, 24; and H 60-6909, 3. Consequently, data for cultivars CP 72-2086 and H 60-6909 at Ft. Lauderdale were eliminated from further study because inadequate numbers of population estimates were obtained. This left a total of 1,136 population density estimates among the remaining 10 cultivar-inoculum combinations. Similar enumeration data were obtained for 141 of the 165 (85.5%) samples from Canal Point.

Variance and the mean. The variance-mean relationship was examined for 71 data sets, each data set consisting of estimates for pathogen densities in sap extracts from five to 30 stalks of a cultivar sampled during one sampling period at either Ft. Lauderdale or Canal Point. The variance-mean relationship for 68 data sets (three data sets were disregarded because they were obvious outliers) can be expressed as: $\log s^2 = 3.078 + 1.580 \log \bar{x}$ ($P < 0.0001$, $R^2 = 0.89$). Because effects due to location (Ft. Lauderdale versus Canal Point) are confounded with effects due to crop (plant versus stubble crop) or year (1983 versus 1984), separate regression analyses were also made for each location-crop-year combination (Table 1). The only significant difference among the regressions was between the intercept estimates for the two crops at Ft. Lauderdale. Covariance analysis of the Ft. Lauderdale data (Table 2) indicated that cultivar and crop (or year) effects but not the censoring effect (33 of the 58 data sets were censored in that numbers of the pathogen were too low for enumeration in at least one of the sap samples) significantly affected the variance-mean relationship. However, cultivar and crop effects did not substantially influence the overall variance-mean relationship, as indicated by their relatively low *F* statistics when compared to both those for mean population density and the statistical model. Because significant ($P < 0.05$) interactions between mean pathogen density and either crop, cultivar, or censoring effects were not indicated, the variance-mean ratio appears to be reasonably constant. Thus, a single transformation to stabilize the variance should be generally applicable.

Variance stabilizing transforms. The transformation, $z = x^p$, where x equals the original number, z equals the transformed value, and $p = 1 - b/2$, has been found to stabilize variances (21), where b is the slope of the regression of log variance on log mean (Table 1). The value of the exponent, p, is 0.2 for the *C. x.* subsp. *xyli* data using the approximation, $b = 1.6$. This suggests that the appropriate transformation is between the logarithmic ($p = 0$) and the square-root ($p = 0.5$) transformations. Transformation success may be measured either as a decrease in correlation, *R*, between the variance and mean, or as a decrease in the range of sample variance (13). The latter may be assessed with the statistic, $\zeta = \ln \rho / 2$, where ρ is the ratio of maximum to minimum variance (9,25). ζ is a normally distributed variable with variance, $\sigma^2 = 1/df$, where df is the degrees of freedom of the sample variance, in this case averaging 16.32 for the 68 data sets. The expected value for $\ln \rho$ from 68 samples, each having a normal distribution, is $\sigma \sqrt{68}$ (16), so that $\zeta = \sqrt{68/16.32}/2 = 1.02$. An iterative computer search produced values of 0.252602 and 1.564×10^7 for the constant, c, of the transformations $z = x^c$ and $z = \log(x + c)$, such that the overall

TABLE 1. Relationship between log variance, y, and log mean, x, for population density estimates of data sets[a]

Location	Crop	Year	n[b]	$\bar{y}$[c]	$\bar{x}$[c]	a ± SE		b ± SE		R^2	t[d]
Ft. Lauderdale	Plant	1983	30	15.63	8.00	3.746	0.730	1.485	0.091	0.90	16.3
Ft. Lauderdale	Stubble	1984	28	15.67	7.89	2.402	0.524	1.682	0.066	0.96	25.4
Canal Point	Plant	1984	10	15.49	7.89	3.192	3.225	1.558	0.408	0.65	3.8

[a] Model is $y = a + bx$.
[b] Number of data sets.
[c] Bar indicates mean of all data sets.
[d] Students *t* for slope; all values highly significant ($P < 0.01$).

correlation between sample variance and mean are zero for the transformed data. Value of the ζ statistic for the exponentially and logarithmically transformed data were 1.17 and 1.43, respectively, suggesting that the exponential transformation stabilizes the variance more effectively than the logarithmic transformation. Consequently, the transformation, $z = x^{0.25}$, which is equivalent to a quartic-root transformation, was selected for further use.

Distribution of population density estimates. The Shapiro-Wilk test indicates that 31 (43.7%) of the 71 data sets are not normally distributed and that the quartic-root transformation reduces this number to 6 (8.5%). When only uncensored data sets are considered, 13 (37.1%) of these 35 data sets are not normally distributed, and the quartic-root transformation reduces this number to 3 (8.6%). Virtually the same results were obtained with the Q-Q correlation test for normality. Because skewedness can greatly affect the validity of the mean as a measure of central tendency, this parameter was also examined. Before transformation, 68 (95.8%) of the 71 data sets have density estimates skewed in a positive direction. After transformation, 29 (40.8%) data sets are skewed in a positive direction. The mean value of the g_1 statistic for skewedness (20) is 1.30 ± 0.10 ($\pm$ standard error) and -0.09 ± 0.10 for nontransformed and transformed data, respectively. Thus, the means of nontransformed data generally overestimate the central tendency of the individual populations and the quartic-root transformation corrects for this discrepancy.

Temporal distribution of the pathogen in different cultivars. A relationship existed between *C. x.* subsp. *xyli* density and sampling date at Ft. Lauderdale and this relationship was influenced by the host cultivar (Table 3). Because interactions between main effects and quadratic effects contributed very little to the overall relationship, most of the variation in population density as affected by cultivar can reasonably be expressed as a series of parallel linear regressions for each crop (Table 4). Thus, pathogen densities generally increased at a similar rate in the third internode of stalks when the crops were sampled, whereas differences in pathogen density remained somewhat constant among cultivars. The significant interaction between time and cultivar for the stubble crop was due to abnormally low density estimates for two stalk samples of one cultivar, CP 72-1210, from one block on the last sampling date and was discounted as possible sampling error.

Variance component analyses. Values of the *F* statistics (1 and 17) obtained from the analyses of variance for nonadditivity (Tukey's test) were 0.00024–3.29 for all six sampling dates at Ft. Lauderdale and were not significant ($P > 0.05$) when means of quartic-root transformed population density estimates for the cultivars in each block were examined. Thus, partitioning of the variance into additive terms should be meaningful. Results of the variance component analyses for pathogen density estimates from the plant crop at Ft. Lauderdale are given in Table 5. The percentage of the variances accounted for by the different effects was similar for both the October and December sampling dates, but unlike these sampling dates, essentially no row effect was detected for the January sampling date.

Pathogen populations as related to planting site and sugarcane yields. The mean population density estimates for cultivars at Canal Point (Table 6) were significantly correlated ($P < 0.01$) with those from both the plant and stubble crops of the same nine cultivars at Ft. Lauderdale (Table 4); the values of correlation coefficient (R) are 0.81 and 0.79 for the respective comparisons. The means for the two crops at Ft. Lauderdale are even more correlated ($R = 0.95$; $P < 0.0001$), as might be expected. Results of the yield study at Canal Point are given in Table 6. A significant reduction ($P < 0.05$) in the mean stalk weights due to RSD was observed for cultivars CP 53-1, CP 59-22, CP 63-588, CP 43-47, CP 44-101, and CP 72-1210. The mean pathogen density estimates for the 11 cultivars at Canal Point were significantly correlated ($R = 0.81$; $P < 0.01$) with mean differences in cultivar yield (kilograms per plant) due to RSD.

TABLE 2. Analysis of covariance with respect to the effects of cultivar, crop, and censoring of data on the relationship between the log variance and log mean of population density estimates for data sets at Ft. Lauderdale, FL

Source of variation	df	SS	MS	F	P	R^2
Model[a]	12	42.806	3.567	87.72	0.0001	0.96
Mean density	1	41.134	41.134	1011.50	0.0001	
Cultivar	9	0.859	0.095	2.35	0.0289	
Crop	1	0.753	0.753	18.52	0.0001	
Censoring	1	0.060	0.060	1.49	0.2290	
Error	45	1.830	0.041			
Total	57	44.636				

[a] Refined model; no significant interactions ($P < 0.05$) between main effects were found using a complete model (analysis not shown).

DISCUSSION

Although attempts were made to perpetuate infected plants by vegetative propagation, some of the resulting plants were either not infected initially or became free of the pathogen between the plant crop and first stubble crop. This situation appeared to be directly related to cultivar resistance to RSD because many of these "noninfected" plants (22 of the 34 plants at Ft. Lauderdale and seven of the nine plants at Canal Point) were of cultivars CP 72-2086 and H 60-6909, which were the most resistant of the 11 cultivars selected for use in this study. The possibility that recovery

TABLE 3. Covariance analysis by crop of population densities of *Clavibacter xyli* subsp. *xyli* in sap extracts from 10 cultivar-treatment combinations (nine cultivars, with one cultivar having been inoculated by two different methods) with days after 1 January for each growing season at Ft. Lauderdale, FL, treated as a covariate[a]

Source of variation	Plant crop				First stubble crop			
	df	SS	MS	F[b]	df	SS	MS	F[b]
Model	49	89668	1830	23.55***	49	126015	2572	12.21***
Block (B)	2	237			2	3640		
Cultivar (C)	9	79471	8830	55.86***	9	107743	11971	47.12***
C error (B × C)	18	2846	158		18	4573	254	
Days (D)	1	5501	5501	70.79***	1	1894	1894	8.99**
D × D	1	176	176	2.27	1	691	691	3.28
D × C	9	741	82	1.06	9	4070	452	2.15*
D × D × C	9	696	77	0.99	9	3404	378	1.79
Error	40	3109	78		40	8428	211	
Total	89	92777			89	134443		
R^2		0.97				0.94		
C.V. (%)		8.52				15.02		
Mean		103.47				96.67		

[a] A randomized complete block design with replications within each plot (block-cultivar combination) was used. Analyses made using plot means for quartic-root transformed population density estimates and for days after 1 January.

[b] *F* test, one significance indicated as: * = $P < 0.05$; ** = $P < 0.005$; *** = $P < 0.001$.

from infection might be an important consideration in studies on RSD has not been addressed but could have a significant effect on the interpretation of experimental results.

The combined results from repeatedly sampling each plant at Ft. Lauderdale were used to identify plants that were not infected, and these plants were subsequently eliminated from any further consideration. Nevertheless, due to the lower limit of resolution of the FADCF technique, a dilemma still exists as to whether *C. x.* subsp. *xyli* was present when it was not detected in sap extracts from particular plants. Furthermore, when only a few fluorescing particles were observed in a sap preparation, it was sometimes difficult to determine whether these were pathogen cells or contaminating plant debris, such as autofluorescing pieces of xylem wall material. Therefore, by setting a minimum count level (one cell per microscope field) for enumeration of the pathogen, the effect of any aberrant counts was reduced. Also, a minimum level of accuracy for individual population density estimates was set, because the accuracy of the estimates obtained by this procedure increases with the number of cells counted (2). Although for practical reasons the number of microscope fields in which cells were enumerated in each sample preparation was limited to a maximum of 15, the accuracy of density estimates, particularly at lower densities, can be improved by examining more microscope fields. If count data could have been obtained for all sap samples from infected plants, the populations most affected would have been those of cultivars supporting comparatively low densities of *C. x.* subsp. *xyli*. For such censored data, the mean pathogen density estimates might have been lower and the range among estimates would have been greater. This increased variability among estimates might have been offset somewhat by the increase in the number of estimates available for analysis when considering the potential outcome of statistical analyses.

To obviate the overall effect of censored data, data from cultivars CP 72-2086 and H 60-6909 at Ft. Lauderdale, which accounted for 41% of the censored data, were excluded from further analyses. Rouse et al (17) described a mathematical procedure for predicting the mean and variance of censored data sets of epiphytic bacterial densities; however, both a lognormal

TABLE 4. Relationship between density of *Clavibacter xyli* subsp. *xyli* and days after 1 January 1983 for the 1983 plant crop and after 1 January 1984 for the 1984 stubble crop for 10 cultivar-inoculum combinations at Ft. Lauderdale, FL[a]

Cultivar	Plant crop			Stubble crop		
	$\bar{y}$	$\bar{x}$	b_o[b]	$\bar{y}$	$\bar{x}$	b_o[b]
CP 53-1-R	169.1	344.8	100.6	170.1	235.1	145.9
CP 53-1	137.1	333.3	70.8	127.2	234.7	103.0
CP 59-22	109.2	337.3	42.1	113.7	235.1	89.5
CP 63-588	105.4	341.0	37.7	106.9	235.2	82.7
CP 43-47	102.6	341.9	34.6	111.0	234.1	86.9
CP 72-1210	106.0	337.3	38.9	87.1	235.4	62.9
CP 74-2005	88.3	342.6	20.1	68.0	236.2	43.7
CP 65-357	82.3	343.0	14.0	54.4	235.7	30.1
CP 44-101	76.9	332.3	10.8	77.7	234.3	53.6
CP 70-1133	59.1	332.7	−7.1	52.7	235.1	28.5

[a] The model is $y = b_o + b_1x$, where y is quartic-root density, b_o is the intercept after adjustment for each cultivar coefficient, b_1 is the coefficient for the covariate days, and x is days. The coefficient, b_1, is equal to 0.199 for the plant crop and 0.103 for the stubble crop.

[b] The complete covariance model, $y = a + bC + b_1x$, was reduced such that $b_o = a + bC$, where the constant, a, is the intercept, and the constant, b, is one of 10 coefficients for cultivar multiplied by a (0,1) choice for C for each cultivar. All coefficients and intercept parameters for the complete model were highly significant ($P < 0.002$) and $R^2 = 0.92$ and $R^2 = 0.84$ for the plant and stubble crops, respectively.

TABLE 5. Variance component analysis of *Clavibacter xyli* subsp. *xyli* population densities in sap extracted from sugarcane stalks sampled in the plant crop at Ft. Lauderdale, FL[a]

Source of variation	Sampling date											
	18 October 1983				2 December 1983				22 January 1984			
	df	SS	Variance component	%	df	SS	Variance component	%	df	SS	Variance component	%
Row[b]	18	15696	62.26	16.6	18	16663	61.86	15.3	18	8276	0.64	0.2
Plant	99	35936	53.14	14.2	104	41687	64.20	15.9	101	45537	135.34	41.5
Error	122	31690	259.75	69.2	124	34417	277.55	68.8	122	23226	190.38	58.3

[a] The SAS Varacomp procedure was used for the analysis. Block and cultivar effects were considered fixed.

[b] The row effects are the same as the interaction effects for blocks and cultivars.

TABLE 6. Mean population densities of *Clavibacter xyli* subsp. *xyli* in sap extracts from sugarcane stalks of different cultivars grown at Canal Point, FL, and the effects of the pathogen on yield of the cultivars[a]

Cultivar	Mean population[b] (cells/ml)	Mean weight (kg/plant)[c]		Difference in mean weight	
		Noninfected	Infected	(kg)	(%)[d]
CP 53-1	5.18×10^8 a	19.33	15.69	3.64 abc	23.2
CP 59-22	1.11×10^8 b	13.51	9.35	4.16 a	44.5
CP 63-588	8.90×10^7 b	14.34	11.19	3.15 abc	28.1
CP 43-47	7.34×10^7 b,c	16.77	12.97	3.80 ab	29.3
CP 44-101	4.75×10^7 c,d	17.14	15.34	1.80 cd	11.7
CP 72-1210	4.55×10^7 c,d	15.59	13.40	2.19 bcd	16.4
CP 74-2005	4.55×10^7 c,d	13.05	12.11	0.95 de	7.8
CP 70-1133	4.47×10^7 c,d	14.48	13.84	0.64 de	4.6
CP 65-357	2.43×10^7 d,e	16.77	16.01	0.76 de	4.8
CP 72-2086	1.55×10^7 e	16.00	14.89	1.11 de	7.5
H 60-6909	1.94×10^5 f	12.32	12.68	−0.36 e	−2.9

[a] Means in the same column followed by the same letter are not significantly different: Waller-Duncan *k*-ratio *t* test; *k*-ratio = 100.

[b] Values are back-transformed from the means of quartic-root transformed data. Each mean represents data for 15 stalks, except for CP 74-2005, CP 72-2086, and H 60-6909, for which samples from nine, seven, and five stalks, respectively, had cell counts above the minimum (one cell per field) set for enumeration (the pathogen was detected in a total of 13, 12, and 11 stalks, respectively). One stalk per plant was sampled, and only 15 of the 29 plants per treatment were examined.

[c] Each mean is for 29 plants.

[d] Values expressed as the percentage of increase in yield when the pathogen was absent.

frequency distribution and censoring are assumed. These assumptions may not be warranted for some *C. x.* subsp. *xyli* data, not only because distributions may not be lognormal, but also because infection cannot always be assumed. Repeated sampling to verify infection by *C. x.* subsp. *xyli* may not be practical in many situations. In the context of screening for RSD resistance, failure to successfully inoculate a particular genotype takes on added significance, because resistance to infection by *C. x.* subsp. *xyli* in sugarcane is inversely correlated with yield reduction in infected plants due to RSD (8). Consequently, inoculum dose effects as well as with pathogen density measurements may need to be considered when selecting for RSD resistance.

A mathematically equivalent model to the linear regression model used to describe the variance-mean relationship of the *C. x.* subsp. *xyli* data is $s^2 = a\bar{x}^b$. Taylor (23,24) hypothesized that the constant, a, is largely a sampling factor and the constant, b, provides a species-specific index of aggregation within the population. The observed approximate value, b = 1.6, suggests a moderately contagious distribution of the *C. x.* subsp. *xyli* population. Variance-mean relationships and nonnormality are common for natural populations of many organisms; fortunately, data transformations often permit effective use of parametric statistics in the analysis of such data. In this study, a quartic-root transformation of population density estimates frequently normalized data sets and eliminated variance-mean relationships. Thus, the quartic-root transformation appears to be generally suited for the analysis of *C. x.* subsp. *xyli* populations using parametric statistics.

Our results indicate that significant differences in the size of *C. x.* subsp. *xyli* populations existed in third internode of stalks among sugarcane cultivars, and furthermore, these differences remained much the same throughout the period of time sampled in each crop. However, the variability associated with measuring pathogen density estimates decreased as the growing seasons progressed. Also, pathogen population sizes increased with time, making it easier to measure them and thus, reducing the amount of censored data. This may account for some of the decrease in variability of the data with time. Overall, the coefficients of variation associated with individual data sets decreased as mean pathogen density estimates increased, even though means increased together with variances. This is indicated by the ratio of the log variance to the log mean being approximately 1.6 (the value of the slope, b, of the linear regression model for the variance-mean relationship). Larger ratios would have been observed had the coefficient of variation remained constant (b = 2) or increased (b > 2) with mean population densities.

Assuming additivity, as indicated by the results of Tukey's test, the variance components associated with the sample mean square given in Table 5 can be used to examine the effects of different levels of replication, as follows:

$$S_{\bar{x}}^2 = \frac{S_r^2}{n_r} + \frac{S_p^2}{n_r n_p} + \frac{S_s^2}{n_r n_p n_s}$$

where $S_{\bar{x}}^2$ is the variance of the mean population density estimate; S_r^2, S_p^2, and S_s^2 are the variance components for row, plants, and determinations within plants; and n_r, n_p, and n_s are the numbers of replications at these levels, respectively (19). Variation among stalks and those associated with the enumeration of the pathogen, in addition to random error, are represented by the variance component for determinations within the plants. Preliminary experiments indicated that as much variability in density estimates was associated with stalks within plants as between plants (*unpublished data*). Additionally, relatively little variability in density estimates was found in an earlier study (2) when subsamples of individual sap extracts were examined using the FADCF technique.

If cost estimates for the different levels of replication were available, these estimates and the variance component estimates could be used together to predict the most economical sampling scheme, within practical limitations, for obtaining a specified variance of the mean population density (19). Within cost restrictions, the maximum variance tolerable would depend on both the magnitude of the difference in population size relative to a given degree of susceptibility to RSD and the desired level of discrimination between resistant and susceptible cultivars. For example, given that the cost associated with individual determinations within plants is relatively much greater than the costs for the other levels of replication and that a least significant difference (LSD) of 15 quartic-root density units at $P < 0.05$ is required to obtain the desired level of discrimination between mean density estimates for cultivars, using the variance component estimates in Table 5, it can be predicted that the most efficient and economical sampling procedure for all sampling dates would involve replicating cultivars in 14–17 blocks, depending on the sampling date, with no replication at the plant or within plant levels. Because little variation due to blocks was detected for the January sampling date, replication at the block or plant level or both to attain the same overall level of replication should produce virtually the same level of discrimination; however, replication at the block level is more efficient for the other sampling dates because of the greater variation among blocks.

Consistently lower populations of the pathogen were found in CP 53-1, which had been heat-treated and then inoculated with the F1 strain of *C. x.* subsp. *xyli*, than in the same cultivar, which had not been heat-treated and then inoculated with crude juice extracts from infected sugarcane. These differences were not substantial enough to affect the ranking of the cultivar with respect to other cultivars on the basis of pathogen densities in this study. Nevertheless, they do suggest that host treatment or pathogen source can have significant effects on the overall population densities observed in sugarcane.

Yield data obtained from the Canal Point planting represent our efforts to obtain the first information on the effects of RSD on different sugarcane cultivars when pure cultures of *C. x.* subsp. *xyli* are used for inoculum and the presence or absence of the pathogen is directly determined for a representative number of plants. Pathogen density in basal stalk internodes was directly correlated to the susceptibility of cultivars to RSD at Canal Point, agreeing with the observations of others (1,7,12). Although the pathogen was not detected in some of the plants of cultivars CP 74-2005, CP 72-2086, and H 60-6909 that were considered infected, the overall ranking of cultivars on the basis of apparent susceptibility to RSD was probably not greatly affected by this discrepancy.

The R^2 values for the correlation between pathogen population densities in nine cultivars at Canal Point with those of the same cultivars in both the plant crop ($R^2 = 0.66$) and stubble crop ($R^2 = 0.62$) at Ft. Lauderdale indicate the dominance of genetic effects over environmental and random effects on this parameter. Furthermore, the fact that correlations between yield reduction due to RSD and pathogen density have consistently been found in Louisiana (7,12), South Africa (1), and now Florida suggests that data on RSD resistance in specific cultivars based solely on relative pathogen population densities will be useful at different geographic locations. Thus, the generally consistent nature of the relationship between pathogen density and yield reduction and the apparent absence of strict limitations with respect to time and geographic location would seem to suggest that screening procedure for RSD resistance in sugarcane based on relative densities of the pathogen might be implemented with a reasonable expectation for success.

LITERATURE CITED

1. Bailey, R. A. 1977. The systemic distribution and relative occurrence of bacteria in sugarcane varieties affected by ratoon stunting disease. Proc. S. Afr. Sugar Technol. Assoc. Ann. Congr. 51:55-56.
2. Davis, M. J. 1985. Direct-count techniques for enumerating *Clavibacter xyli* subsp. *xyli* which causes ratoon stunting disease of sugarcane. Phytopathology 75:1226-1231.
3. Davis, M. J., Gillaspie, A. G., Jr., Harris, R. W., and Lawson, R. H. 1980. Ratoon stunting disease of sugarcane: isolation of the causal

bacterium. Science 210:1365-1367.
4. Davis, M. J., Gillaspie, A. G., Jr., Vidaver, A. K., and Harris, R. W. 1984. *Clavibacter*: a new genus containing some phytopathogenic coryneform bacteria, including *Clavibacter xyli* subsp. *xyli* sp. nov., subsp. nov. and *Clavibacter xyli* subsp. *cynodontis* subsp. nov., pathogens that cause ratoon stunting disease of sugarcane and Bermudagrass stunting disease. Int. J. Syst. Bacteriol. 34:107-117.
5. Dean, J. L. 1983. Single-stool plots for estimating relative yield losses caused by ratoon stunting disease of sugarcane. Plant Dis. 67:47-49.
6. Gillaspie, A. G., Jr., Davis, R. E., and Worley, J. F. 1973. Diagnosis of ratoon stunting disease based on the presence of a specific microorganism. Plant Dis. Rep. 57:987-990.
7. Gillaspie, A. G., Jr., Flax, G., and Koike, H. 1976. Relationship between numbers of diagnostic bacteria and injury by ratoon stunting disease in sugarcane. Plant Dis. Rep. 60:573-575.
8. Harrison, N. A., Davis, M. J., and Dean, J. L. 1986. Infectivity titrations of *Clavibacter xyli* subsp. *xyli* and sugarcane cultivars differing in susceptibility to ratoon stunting disease. Plant Disease 70:556-558.
9. Hayman, B. I., and Lowe, A. D. 1961. The transformation of counts of *Aphis fabae* Scop. on beans. J. Appl. Biol. 65:181-189.
10. Hirano, S. S., Nordheim, E. V., Arny, D. C., and Upper, C. D. 1982. Lognormal distribution of epiphytic bacterial populations on leaf surfaces. Appl. Environ. Microbiol. 44:695-700.
11. Hughes, C. G. 1974. The economic importance of ratoon stunting disease. Proc. Int. Soc. Sugar Cane Technol. 15:213-217.
12. Koike, H., Gillaspie, A. G., Jr., and Benda, G. T. A. 1982. Cane yield response to ratoon stunting disease. Int. Sugar J. 84:131-133.
13. Logan, P. A. 1980. Spatial distribution of cereal leaf beetle (*Oulema melanopus* (L)) eggs and larvae and treatment of count data. Environ. Entomol. 9:186-189.
14. Loper, J. E., Suslow, T. V., and Schroth, M. N. 1984. Lognormal distribution of bacterial populations in the rhizosphere. Phytopathology 74:1454-1460.
15. Maramorosch, K., Plavsic-Banjac, B., Bird, J., and Liu, L. J. 1973. Electron microscopy of ratoon stunted sugarcane: microorganisms in xylem. Phytopath. Z. 77:270-273.
16. Masuyama, M. 1957. The use of the sample range in estimating the standard deviation or the variance of any population. Sankhya 18:159-162.
17. Rouse, D. I., Nordheim, E. V., Hirano, S. S., and Upper, C. D. 1985. A model relating the probability of foliar disease incidence to the population frequencies of bacterial plant pathogens. Phytopathology 75:505-509.
18. Shapiro, S. S., and Wilk, M. B. 1965. An analysis of variance test for normality (complete samples). Biometrika 52:591-611.
19. Snedecor, G. W., and Cochran, W. G. 1973. Statistical Methods. The Iowa State University Press, Ames.
20. Sokal, R. R., and Rohlf, F. J. 1969. Biometry. Freeman and Co., San Francisco.
21. Southwood, T. R. E. 1966. Ecological Methods. Chapman and Hall, London.
22. Steindl, D. R. L. 1961. Ratoon stunting disease. Pages 433-459 in: Sugarcane Diseases of the World, vol. 1. J. P. Martin, E. V. Abbott, and C. G. Hughes, eds. Elsevier Publishing Co., Amsterdam.
23. Taylor, L. R. 1961. Aggregation, variance and the mean. Nature (London) 189:732-735.
24. Taylor, L. R. 1965. A natural law for the spatial disposition of insects. Proc. XII Int. Congr. Entomol. London, 1964.
25. Taylor, L. R. 1970. Aggregation and the transformation of counts of *Aphis fabae* Scop. on beans. J. Appl. Biol. 65:181-189.
26. Teakle, D. S., Smith, P. M., and Steindl, D. R. L. 1973. Association of a small coryneform bacterium with the ratoon stunting disease of sugarcane. Aust. J. Agric. Res. 24:869-874.
27. Todd, E. H. 1960. The ratoon stunting disease of sugarcane and its control in Florida. USDA Crops Res. ARS 34-12.