Colonization of Vascular Tissues by *Clavibacter xyli* subsp. *xyli* in Stalks of Sugarcane Cultivars Differing in Susceptibility to Ratoon Stunting Disease

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ABSTRACT

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A novel tissue blot enzyme immunoassay was developed to identify vascular bundles colonized by *Clavibacter xyli* subsp. *xyli* in stalk internodes of sugarcane with ratoon stunting disease (RSD). Numbers of infected bundles in tissue cross sections declined progressively with increasing sampling distance of internodes from the base of stalks in cultivars CP 70-1133, CP 44-101, and CP 72-1210. CP 70-1133, the least susceptible to RSD of the three cultivars, contained significantly fewer infected bundles than did CP 72-1210 and CP 44-101. Conversely, infected bundles were most numerous in CP 72-1210, the most susceptible cultivar. Numbers of infected bundles in comparable basal internodes of 10 sugarcane cultivars were positively correlated with population densities of *C. x.* subsp. *xyli* in sap extracts from these tissues. Pathogen densities in sap from different cultivars have been shown to be correlated directly with cultivar susceptibility to RSD; results of the present study indicate that cultivar resistance to RSD also is associated with reduced pathogen colonization of vascular tissues.

Ratoon stunting disease (RSD) of sugarcane (interspecific hybrids of *Saccharum*), caused by the xylem-inhabiting, coryneform bacterium *Clavibacter xyli* subsp. *xyli* (8), is widely regarded as the most important disease affecting commercial sugarcane production (11). No sugarcane cultivars are immune to infection by *C. x.* subsp. *xyli*; however, resistance to RSD has been recognized in some cultivars (14,15,19). Although the basis for this resistance has not been clearly elucidated, studies on water flow through single-node cuttings (14,15) and movement of India ink particles in the xylem of sugarcane stalks (15) suggest that features of innate vascular structure at the stalk nodes may play an important role by restricting the intraxylar spread of the pathogen in resistant plants. These features include a more profuse branching of the metaxylem (14) and fewer xylem vessels that pass directly through stalk nodes without terminating (15).

Studies on the population dynamics of *C. x.* subsp. *xyli* in sugarcane have provided evidence for both spatial and temporal differences in pathogen population densities within cultivars (6,10). Typically, bacterial populations in sap extracts of all cultivars attained their greatest densities first in basal portions of more mature, systemically infected stalks (6). Also, mean pathogen densities in tissue diffusates, extracted sap (2,6) and in crude juice from milled cane (10) from comparable internodes of different cultivars were inversely correlated with cultivar resistance to RSD. These findings were consistent for both the plant crop (first crop) and first ratoon crop (second crop or first stubble crop) (6). The present study was undertaken to examine the patterns of vascular colonization of sugarcane cultivars by *C. x.* subsp. *xyli* and to assess whether cultivar susceptibility to RSD is associated with differences in numbers of infected vascular bundles within these plants. A modified immune-blot assay for detecting and enumerating vascular bundles containing *C. x.* subsp. *xyli* also is described.

MATERIALS AND METHODS

Plant material. Healthy and diseased plantings of 10 sugarcane cultivars (CP 72-1210, CP 44-101, CP 70-1133, CP 53-1, CP 65-357, CP 63-588, CP 72-2086, CP 74-2005, CP 43-47, and CP 59-22), which differ in susceptibility to RSD on the basis of yield reduction estimates (6,9), were established and maintained in experimental plots at the Ft. Lauderdale Research and Education Center, as described previously (6). Plants were propagated from single-node cuttings of stalks previously heat treated (16) to eliminate any naturally occurring RSD infections. Cuttings were inoculated, immediately before planting, by immersing them in 7-day-old RSD broth (8) cultures containing about 10^8 cells per milliliter of the F-1 strain (5) of *C. x.* subsp. *xyli*.

Plants were sampled during the second ratoon (third year or second stubble) crop by severing mature stalks at the soil line using pruning shears. Stalks were washed with soap and running water to remove any surface grime. After surfaces air dried, internodes 3, 5, 7, and 9 were excised from stalks and each halved transversely. An 18-mm-diameter cork borer was used to remove a core of tissue (20 mm in length) from centrally located tissues within each internode half. The cores were used in subsequent analyses.

Detection and quantitation of infected vascular tissue. Ends of excised cores from the acropetal one-half of each internode were trimmed flat, and three sequential, 5-mm-thick cross sections were removed with a razor blade from the basal end. Each section was placed onto a 0.45-μm-pore membrane filter disk (20 mm diameter) cut from 15 × 9.2 cm sheets of pure nitrocellulose (Trans-Blot Transfer Medium, Bio-Rad, Richmond, CA). Filter disks had previously been seated in clear plastic culture tube caps (22 mm inside diameter) fitted with an 18 × 22 mm polystyrene plug to support both the filter and a layer of 10 disks of paper toweling immediately below the filter. Caps with tissue sections were placed in 50-ml, conical-bottom, plastic centrifuge tubes, and the vascular contents of sections were extracted onto the filter by centrifugation at 1,600 *g* for 15 min. The paper towelling served as a cushion for filters during centrifugation and absorbed any sap released from the tissue sections, and cells of the pathogen were retained by the filters. After centrifugation, filters were carefully removed from tissue sections and placed in 2.4 × 1.7 cm wells of multiwell tissue culture plates (Flow Laboratories Inc., McLean, VA), then air dried overnight at 4 C before serological staining. Corresponding tissue sections were placed onto microscope slides, moistened with a few drops of 2.5% glutaraldehyde in 0.05 M phosphate buffer, pH 8.0, and stored at 4 C in petri dishes for subsequent enumeration of total vascular bundles per section.

A modified indirect enzyme-linked immunosorbent assay (ELISA) procedure (17) was used to stain *C. x.* subsp. *xyli* cells deposited onto filters during centrifugation of tissue sections. Antiserum against the F-1 strain of *C. x.* subsp. *xyli* from culture with specificity for *Clavibacter xyli* was produced, and the IgG was purified by methods described previously (5). The purified IgG (1 mg protein/ml) was filtered through a 0.2-μm Acrodisc filter apparatus (Gelman, Ann Arbor, MI) immediately before use and then diluted 1:400 with ELISA buffer (0.01 M phosphate buffered saline [PBS], pH 7.2, 2% polyvinylpyrrolidone-10, 0.5% bovine serum albumin, 0.2% Tween-20, 0.02% sodium azide). Sufficient volume (0.5 ml) of the diluted IgG was added to wells of tissue culture plates to totally immerse the filter disks. After incubation for 1 hr at 25 C, the filters were removed and washed with three changes (5 min each) of ELISA washing solution (0.01 M PBS, pH 7.2, 0.2% Tween-20, 0.02% sodium azide). Next, filters were incubated 1 hr at 25 C in goat antirabbit IgG conjugated with alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) diluted 1:1000 with ELISA buffer.

After washing as before with ELISA washing solution, antigen-antibody complexes were detected by a color change after incubation 30 min in the dark at 25 C in a substrate solution. The substrate solution was prepared by dissolving 0.15 g naphthol-AS-phosphate (sodium salt) in 2.5 ml *N,N*-dimethyl-formamide and combining this with 500 ml 0.2 Tris buffer, pH 9.1. A diazonium salt (1 mg Fast Blue BB/ml) and 5 μl of 0.1 m $MgCl_2$/ml were added to the substrate solution immediately before use. The enzyme-substrate reaction yielded a dark blue precipitate that could be observed on the nitrocellulose filters. Stained filters were rinsed briefly with distilled water, immersed in a 1% solution of sodium hypochlorite for 30 min, rinsed a second time with distilled water, and allowed to air dry.

Filter disks were examined with a stereomicroscope at 20 ×. Aggregates of *C. x.* subsp. *xyli* cells that appeared as small discrete areas of blue stain on filters were counted. Each aggregate was marked with ink during counting to avoid the possibility of counting the same aggregate more than once. The total number of vascular bundles in each corresponding internode cross section was similarily counted.

In this manner, three stalks from five plants of cultivars CP 72-1210, CP 44-101, and CP 70-1133 were sampled during October 1985. The experiment was repeated during December. During February 1986, all 10 sugarcane cultivars were sampled; however, determinations were limited to the third basal internode of stalks.

Measurement of bacterial populations. Population densities of *C. x.* subsp. *xyli* were estimated by a direct count technique (4). Briefly, sap extracts from tissue cores excised from the basipetal one-half of each internode were collected by centrifugation at 1,600 *g* for 15 min. The bacteria in extracts were stained with a fluorescent antibody, collected on the surface of a membrane filter, and counted using epifluorescence microscopy at 1,200×.

Cross-sectional distribution of infected vascular bundles in stalks. To determine the extent of infestation of vascular tissues by *C. x.* subsp. *xyli* in cultivars CP 72-1210, CP 44-101, and CP 70-1133, two stalks from each of three plants of each cultivar were harvested in January 1986, and internodes 3, 5, 7, and 9 were excised from stalks and halved as described previously. Acropetal internode halves were split lengthwise into four equal-sized pieces with a microtome knife. Three sequential 0.5-mm-thick cross sections were removed from a single quartered internode piece selected arbitrarily from each of the four internode locations sampled within stalks. The vascular contents of tissue cross sections were blotted onto nitrocellulose filters by centrifugation as described above; the filters were stained; and the number of stained spots was determined for the inner, middle, and outer one-third of the cross-sectional area of each wedge-shaped internode section. The total number of vascular bundles was counted in each of the corresponding tissue sections.

RESULTS

Transverse sections of internodes of CP 72-1210, CP 44-101, and CP 70-1133 showed an arrangement of discrete vascular bundles resembling that described by Artschwager (1) for sugarcane (Fig. 1A). The bundles were scattered throughout a parenchymatous ground tissue and were more numerous with increasing proximity to the periphery of internodes. Typically, each bundle contained two large metaxylem vessels separated by smaller, intervening metaxylem elements and protoxylem in which a lacuna was often present. Also, large metaxylem vessels of bundles adjacent to the epidermis were generally much smaller and more variable in size than were those of central bundles. The average diameter from measurements of 100 large metaxylem vessels in peripheral bundles of basal third internodes of CP 72-1210 was 31.5 μm, whereas the equivalent value for vessels of central bundles was 89.8 μm. Metaxylem vessel sizes were similar for all three cultivars.

Tissue blot enzyme immunoassay. Aggregates of *C. x.* subsp. *xyli* cells deposited on nitrocellulose filters were readily located by the tissue blot enzyme immunoassay (TBEIA). Patterns of staining generally consisted of discrete, blue-colored, circular-to-oval areas varying in both size and color intensity. Frequently, stained areas were comparable in size to and often resembled the cross-sectional profile of a vascular bundle in tissue sections (Fig. 1B and C). Also, impressions of the cut surfaces of internode tissues often were observed on filters after centrifugation. Imprints of vascular bundles clearly marked areas of intense staining and also areas where no staining was visible. Similar staining patterns were produced by internode sections from all cultivars tested. Staining of deposits prepared from tissues of noninfected plants was not observed on filters although imprints of vascular bundles were clearly evident. Therefore, each of these stained areas was interpreted as representing an individual, bacteria-infested bundle in the corresponding tissue section.

Occasionally, interspersed between areas of discrete staining, there were areas of slightly more diffuse and less intense staining requiring more careful visual interpretation. These areas appeared to represent a broader deposition of bacteria and resulted, presumably, from the lateral spread of sap containing bacteria between the interface of the tissue section and filter disk during centrifugation. Areas of more diffuse staining were not outlined by imprints of vascular bundles on filters.

The sensitivity of the TBEIA for consistently detecting cell deposits on membranes was greater than 7.07×10^3 cells/mm^2 of nitrocellulose membrane surface (i.e., pathogen population densities greater than 1×10^6/ml of sap extract) as determined by dot ELISA (3) using extracted sap. Therefore, to detect cell deposition in an area equivalent in size to the cross-sectional area of a metaxylem vessel (approximately 0.006 mm^2), at least 46 cells would need to be present on the membrane. Stained deposits of similar dimensions to individual metaxylem vessels were observed

on filters (Fig. 1C).

A light yellow-brown discoloration of nitrocellulose filters was sometimes observed after centrifugation of internode sections excised from close proximity to stalk nodes. Incubation of filters in 1% sodium hypochlorite after staining effectively removed the discoloration and, because bleaching did not appear to affect the stain, the treatment greatly enhanced the staining contrast. Furthermore, the intensity and degree of resolution provided by the staining procedure enabled areas of bacterial deposition to be accurately counted at low magnifications (10–20×) using a stereomicroscope. This permitted the examination of numerous, relatively large cross-sectional areas of stalk tissue for infected bundles.

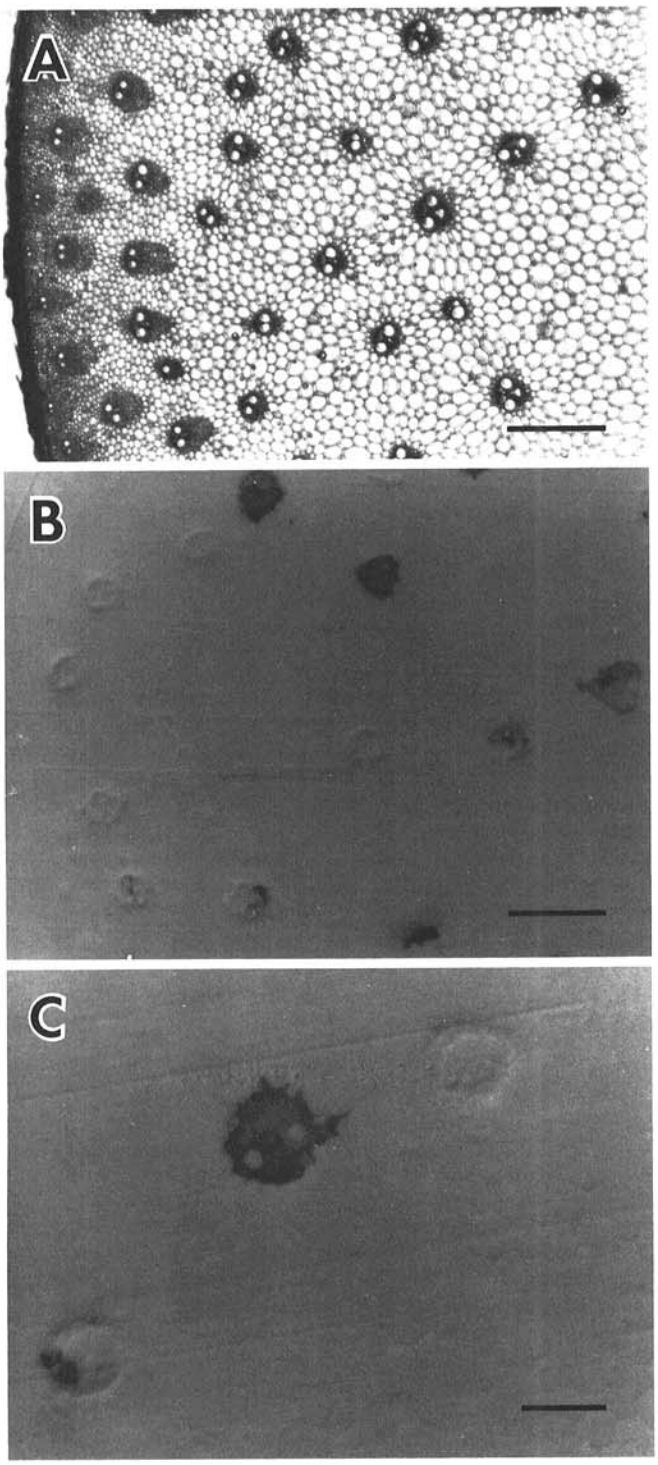

Fig. 1. Detection of vascular bundles containing *Clavibacter xyli* subsp. *xyli* in sugarcane with ratoon stunting disease. **A,** Transverse section of an internode of sugarcane cultivar CP 72-1210 stained with 0.1% safranin showing the cross-sectional profile and arrangement of discrete vascular bundles in both peripheral and more centrally located stalk tissues (scale bar = 0.75 mm). **B,** Appearance of aggregations of *C. x.* subsp. *xyli* cells on nitrocellulose membrane filter disks after their displacement from internode cross sections by centrifugation and indirect enzyme-linked immunosorbent assay staining (scale bar = 0.75 mm). **C,** Discrete area of staining resembling the cross-sectional profile of an individual vascular bundle in conjunction with imprints of adjacent infected and noninfected vascular bundles and associated parenchyma ground tissue on the filter disk surface (scale bar = 0.3 mm).

Distribution of infected vascular bundles. Differences between cultivars CP 72-1210, CP 44-101, and CP 70-1133 were found in both the total number of vascular bundles and bundles containing *C. x.* subsp. *xyli* within stalk internodes (Fig. 2). Tissue cross sections from quartered internodes of CP 70-1133, the least susceptible of the three cultivars, contained significantly greater numbers of vascular bundles at all internode locations sampled as compared to CP 44-101 and CP 72-1210, both of which contained similar numbers (Fig. 2A). By contrast, significantly fewer infected bundles were detected at each location in CP 70-1133 than in CP 44-101 and CP 72-1210. Infected bundles were most numerous in internodes of CP 72-1210, the most susceptible of these cultivars (Fig. 2B).

Infected bundles were scattered throughout the entire cross-sectional area of all internode sections; however, the percentage of infected vascular tissue was greatest within the inner one-third of stalks where bundles were least numerous (Fig. 2C). Conversely, the percentage of infected vascular tissue decreased successively within the middle and outer one-third of stalks although vascular bundles were increasingly more numerous with closer proximity to the stalk epidermis.

Ten cultivars were sampled late in the 1985–1986 growing season, when populations of *C. x.* subsp. *xyli* approach their maximum densities in basal internode tissues (6). The percentages of infected vascular bundles were quite variable and ranged from 68.4% for CP 63-588 to 4.6% for CP 72-2086 (Table 1). When cultivars were ranked according to these percentages, it was found, for most cultivars, that the order corresponded to that for estimates of their relative susceptibility to RSD.

Spatial and temporal distribution of infected vascular bundles. Because cross sections from standard-size (18-mm-diameter) tissue cores from internodes of varying diameters were used in this experiment, percentages of infected vascular bundles were not totally representative of entire internodes because varying amounts of peripheral stalk tissue were discarded when taking cores. Consequently, because infected bundles were most numerous in central internode tissues of the three cultivars examined in greater detail (Fig. 2C), values obtained from cores apparently overestimated the overall percentage of infected bundles in internodes but, nevertheless, reflected those patterns observed when using the more inclusive sections from quartered internodes.

On each sampling date, the percentage of infected bundles detected in cored tissues of cultivars CP 72-1210, CP 44-101, and CP 70-1133 decreased with each successive internode sampled from the base of stalks (Table 2). Infected bundles were most numerous in stalks of CP 72-1210 and least numerous in CP 70-1133 on both sampling dates. Furthermore, the percentage of infected bundles was greater in internodes of all three cultivars on the second sampling date.

Relationship between pathogen densities and numbers of infected bundles. A quartic root transformation was found in a recent study on the population dynamics of *C. x.* subsp. *xyli* in sugarcane to generally normalize density estimates and eliminate the relationship between means and variances (6). Because population density estimates for *C. x.* subsp. *xyli* were obtained in this study in the same manner, quartic root transformations of the estimates were performed prior to analysis of pathogen densities using parametric statistics.

Population density estimates of *C. x.* subsp. *xyli* in sap extracts from internode tissues decreased gradually in cultivars CP 72-1210, CP 44-101, and CP 70-1133 with each successive internode

sampled from the base of stalks and were found to differ significantly between cultivars (Table 2). Consistently greater densities of the pathogen occurred in extracts from stalks of CP 72-1210 and CP 44-101 as compared to CP 70-1133. As population densities declined in internodes of each cultivar, a corresponding decrease in both the number and percentage of infected vascular bundles also was observed. Further analysis of this relationship using data from comparable internodes of 10 sugarcane cultivars (Fig. 3) revealed a high linear correlation ($r = 0.95$; null hypothesis: slope = 0, $P = 0.0001$) between mean number of infected bundles and corresponding pathogen density estimates. A lesser correlation ($r = 0.778$) and significance ($P = 0.0079$) was found, however, between mean percentage of infected vascular bundles and pathogen densities.

DISCUSSION

Patterns of colonization of vascular tissues by *C. x.* subsp. *xyli* in internodes of sugarcane cultivars differing in susceptibility to RSD appeared to be correlated with the maturity of the stalk tissues. Generally, infected vascular bundles were most numerous and pathogen population densities greatest in tissues of lower, more mature internodes. A corresponding decline in both infected bundles and pathogen density estimates was observed within successive internodes from the base of stalks. Also, a gradation of infestation of internode cross sections was evident in each cultivar. Infected vascular tissue was most extensive in the inner, most mature tissues of stalks and least extensive in the outer tissues adjacent to the epidermis. This pattern of vascular colonization persisted in the three cultivars, CP 72-1210, CP 44-101, and CP 70-1133, that were examined in detail although infected vascular tissue and pathogen populations increased in each cultivar with time. Furthermore, when extent of colonization of comparable internodes was examined, there were significant differences between cultivars, and, for most cultivars, the degree of colonization was indicative of their relative susceptibility to RSD.

Although the basis for cultivar differences in colonization by *C. x.* subsp. *xyli* was not investigated in the present study, innate features of vascular anatomy at the nodes of stalks may be involved by restricting the spread of the pathogen in the xylem (14,15). Teakle et al (15) demonstrated that, of 11 cultivars examined,

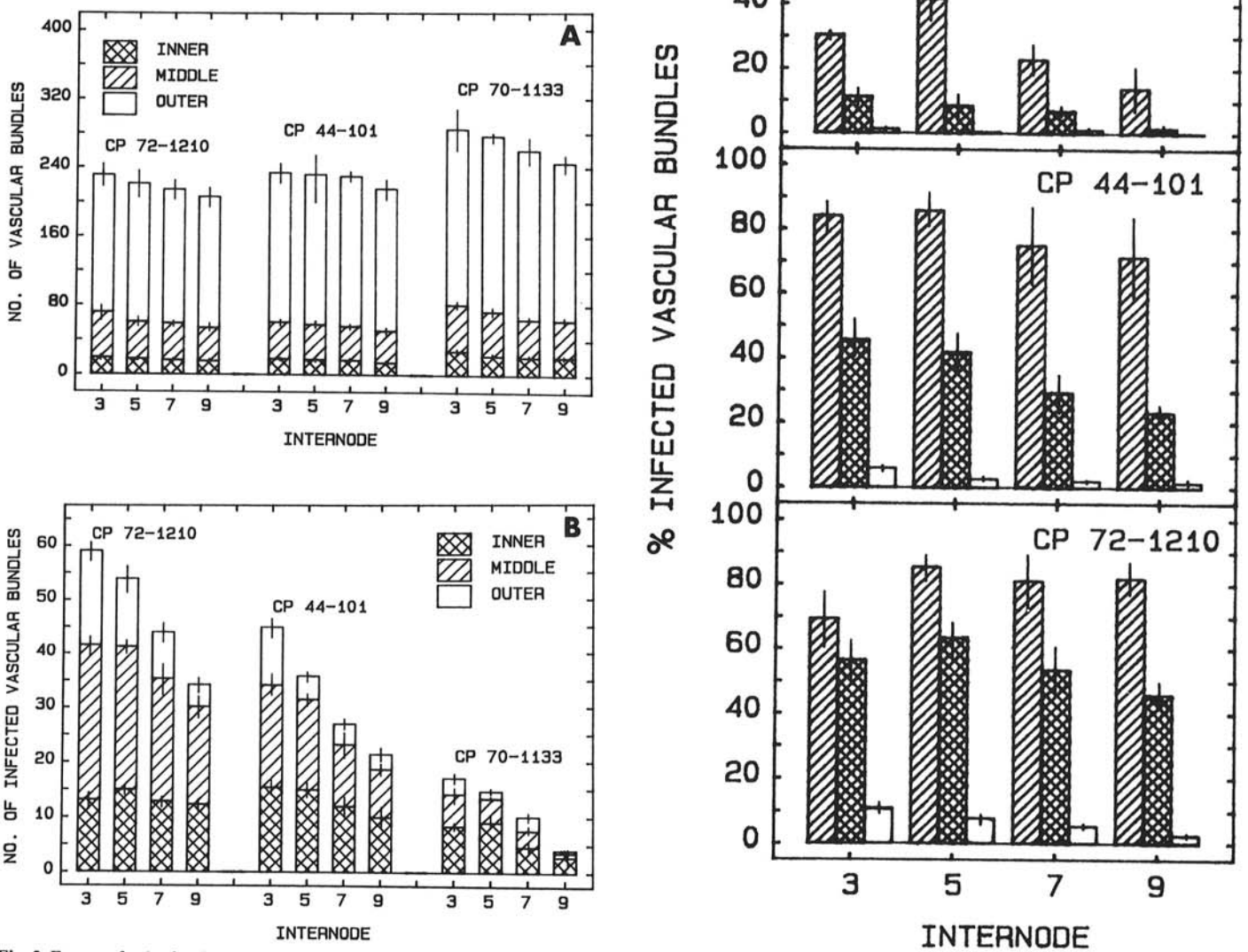

Fig. 2. Extent of colonization of vascular tissues by *Clavibacter xyli* subsp. *xyli* in quartered internode cross sections from various internode locations within stalks of sugarcane cultivars CP 72-1210, CP 44-101, and CP 70-1133 with ratoon stunting disease. **A,** Mean number of vascular bundles in the inner, middle, and outer one-third of stalk sections. Vertical bars indicate 2 standard error of the mean (SEM). **B,** Mean number of infected bundles in the inner, middle, and outer one-third of stalk sections. Vertical bars indicate SEM. **C,** Percent infected bundles in the inner, middle, and outer one-third of stalk sections. Vertical bars indicate SEM.

blackened vascular bundles, which resulted from drawing India ink particles by suction pressure through healthy sugarcane stalk sections, were increasingly less numerous in successive internodes beyond the point of ink introduction. Also, the number of bundles stained after the passage of ink particles through stalk nodes differed substantially amongst cultivars, and these values were negatively correlated with cultivar resistance to RSD. Similarily, the rate of water movement through single-node cuttings of stalks of healthy sugarcane cultivars also was negatively correlated with cultivar resistance to RSD (14,15). Both reduced water flow and limited particle passage within the xylem were attributed to a more profuse branching of metaxylem vessels which resulted in fewer vessels that passed directly through nodes without terminating in resistant cultivars (14).

In the present study, the number of infected vascular bundles in stalk internodes was probably underestimated due to the lower limits of sensitivity of the serological detection technique. Also, occlusion of metaxylem vessels by gels (12) and gums (13,18) has been observed in some cultivars with RSD. The affected vessels, which are discolored, are found most frequently in close proximity to stalk nodes and have been used as a diagnostic symptom of the disease in some cultivars (13), including CP 44-101. Presumably, blockage of vessels by gels and gums could reduce the sensitivity of the TBEIA to detect infected bundles by interfering with deposition of the pathogen onto membrane filters during centrifugation. For this reason, tissue sections were excised from the mid-region of internodes in an attempt to avoid any possible effects due to occlusion products. The likelihood that not all infected bundles were detected, however, was further indicated after an examination of comparable basal internodes of 10 cultivars. Pathogen densities in these tissues were found to vary by as much as 45-fold between cultivars, whereas the percentage of infected bundles differed by as much as 13-fold. Therefore, patterns of vascular infection revealed by the TBEIA probably reflected only the distribution of those bundles containing pathogen cell densities greater than 1×10^6 per milliliter of sap extract.

Diagnosis of RSD and assessment of cultivar susceptibility are made difficult by the nonlethal and almost symptomless nature of the disease. Yield trials comparing healthy and diseased plantings are the only proven method for assessing cultivar reaction to RSD but are both time-consuming and expensive and are rarely used. Consequently, the relative susceptibility of only a few cultivars used in commercial production today are known. A possible alternative approach to evaluating cultivars, based on

TABLE 1. Mean percentage of vascular bundles containing *Clavibacter xyli* subsp. *xyli* in cross sections of central tissues from basal third internodes of stalks of 10 sugarcane cultivars with ratoon stunting disease

Cultivar	No. of stalks[a]	Infected vascular bundles[b] (%)	Yield loss[c] (kg/plant)
CP 63-588	10	68.9 a	2.5
CP 53-1	10	62.4 ab	4.0
CP 59-22	10	51.6 bc	6.4
CP 72-1210	10	49.9 c	2.1
CP 44-101	10	32.3 d	1.8
CP 43-47	10	24.9 de	4.2
CP 74-2005	9	23.4 de	0.9
CP 70-1133	9	16.6 ef	0.9
CP 65-357	10	16.4 ef	1.8
CP 72-2086	5	4.6 f	0.1

[a] Two stalks from five plants of each cultivar were sampled. Determinations of vascular bundles infected with *C. x.* subsp. *xyli* were limited to 18-mm-diameter tissue cores taken from central internode tissues. Only stalks in which infected bundles were detected were used.

[b] Values of mean percent infected vascular bundles followed by the same letter are not significantly different. Waller-Duncan K-ratio *t* test: Kratio = 100 (i.e., alpha level is approximately 0.05).

[c] Published data of Davis et al (7). Values for cultivars, expressed as mean weight loss (kg/plant), represent a compilation of data from single-stool yield trials (9) conducted from 1979 to 1984 at the USDA Sugarcane Field Station, Canal Point, FL, and were used as a best estimate of the relative susceptibility of the test cultivars to ratoon stunting disease.

TABLE 2. Mean percentage of vascular bundles containing *Clavibacter xyli* subsp. *xyli*, and pathogen population densities in sap extracts in centrally located tissues of internodes at various internode locations within stalks of three sugarcane cultivars with ratoon stunting disease sampled on two different dates

Cultivar and date sampled	Internode	Infected vascular bundles[a] (%)	Pathogen populations[b] Cells/ml of sap ($\times 10^7$)
October 30, 1985[c]			
CP 72-1210	3	49.28 ± 3.6	21.59 ± 7.8
	5	33.01 ± 3.1	9.70 ± 2.4
	7	20.62 ± 2.4	5.72 ± 1.6
	9	11.20 ± 1.7	5.48 ± 1.3
CP 44-101	3	23.95 ± 1.5	7.52 ± 1.1
	5	21.27 ± 1.3	7.40 ± 1.3
	7	17.15 ± 1.2	5.13 ± 1.1
	9	10.90 ± 0	3.22 ± 0.4
CP 70-1133	3	8.26 ± 0.8	5.26 ± 0.8
	5	4.48 ± 0.4	2.29 ± 0.5
	7	2.56 ± 0.3	1.17 ± 0.3
	9	1.30 ± 0.2	0.50 ± 0.3
December 14, 1985			
CP 72-1210	3	74.08 ± 3.0	26.58 ± 4.0
	5	57.52 ± 3.7	20.07 ± 4.6
	7	44.72 ± 3.3	13.13 ± 2.5
	9	29.79 ± 2.6	7.79 ± 1.8
CP 44-101	3	39.00 ± 2.3	18.30 ± 4.1
	5	37.25 ± 2.4	15.20 ± 2.9
	7	33.74 ± 2.4	13.33 ± 3.0
	9	31.81 ± 2.5	11.21 ± 2.6
CP 70-1133	3	19.51 ± 2.6	7.76 ± 1.4
	5	12.30 ± 1.2	5.17 ± 1.0
	7	8.59 ± 0.9	2.77 ± 0.7
	9	4.24 ± 0.5	1.08 ± 0.4

[a] Each value represents the mean of cross-sections ± standard error of the mean (SEM) of 18-mm-diameter tissue cores taken from four internode locations within three stalks of five plants of each cultivar on each sampling date.

[b] Population density estimates of *C. x.* subsp. *xyli* per milliliter of sap extract ± SEM as determined by a direct count technique and epifluorescence microscopy at × 1,200.

[c] Mean sampling date of each experiment. All stalks were harvested within 8 to 10 days of each other in each experiment.

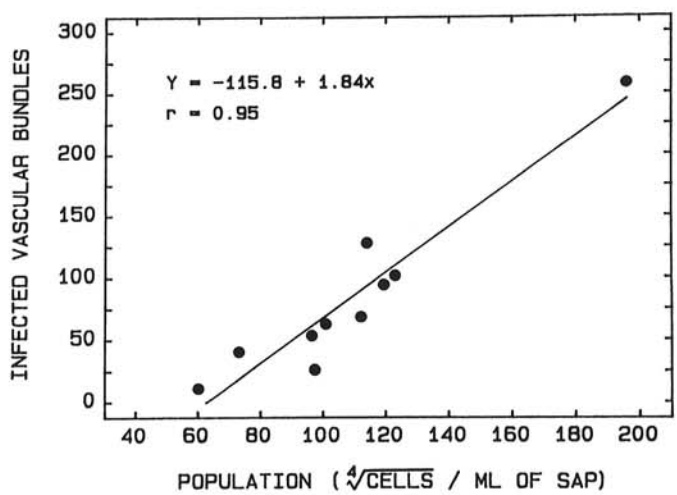

Fig. 3. Relationship between mean number of vascular bundles containing *Clavibacter xyli* subsp. *xyli* and population densities ($\sqrt[4]{}$ cells/ml) of the pathogen in sap extracts from central tissues of third basal internodes of stalks of 10 sugarcane cultivars with ratoon stunting disease.

measurement of pathogen population densities in stalk tissues, has been suggested (6,10). This is because population density estimates from comparable tissues of different cultivars correlate well with yield loss due to RSD (6). Therefore, the high correlation between counts of infected bundles and pathogen densities in extracted sap from comparable tissues of different cultivars in this study is evidence that numbers of infected bundles also may provide a useful indication of cultivar reaction to RSD.

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