Sensitivity of Sugarcane Clones to Toxin from *Helminthosporium sacchari* as Determined by Electrolyte Leakage

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ABSTRACT

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Host-selective toxin from *Helminthosporium sacchari* was processed to a high level of purity and was used in assays of relative sensitivities of sugarcane clones. An assay based on toxin-induced loss of electrolytes from leaf tissue was more precise than were assays based on visible expressions of toxicity. Electrolyte losses from tissues exposed to toxin for 1 hr and monitored for 18 hr equalled those of tissues exposed to toxin continuously for 18 hr. The rate of loss increased for 2–3 hr, then decreased. When toxin concentrations were increased from 0.1 to 0.5 μg/ml, electrolyte loss increased linearly. High concentrations (50 μg/ml) resulted in gradual but complete loss of ability of tissues to retain electrolytes. Sugarcane clone reactions varied from very sensitive (affected by toxin at 0.01 μg/ml) through several intermediate levels to very insensitive (unaffected by at least 100 μg/ml). In general, sensitivity or insensitivity to toxin correlated with susceptibility or resistance of the fungus. However, the correlation did not hold for three clones of 17 tested. The data suggest that toxin may determine pathogenicity to some clones but not to others, and that toxin should be used with caution in screening for disease resistance.

Sugarcane (*Saccharum officinarum* L.) clones vary in reaction to *Helminthosporium sacchari* (Van Breda de Haan) Butler from highly susceptible through several intermediate levels to highly resistant (3,8). Also, the existence of a host-selective toxin from this fungus is well-known (13). If the activity pattern of toxin from *H. sacchari* follows those of several other host-selective toxins (10), there should be a correlation between sensititivy to toxin and susceptibility to the fungus. Correlation of that kind was not detected by Strobel (14), who reported an assay based on the effects of drops of toxin solution on leaves. In preliminary experiments, we found the leaf-drop assay to be inadequate for differentiating clones with intermediate toxin sensitivity. A more precise assay was needed.

Assays based on toxin-induced leakage of electrolyte from tissues have been useful in studies of several host-selective toxins (1,4,10). Byther and Steiner (2) used such an assay to measure seasonal and heat-induced changes in sensitivity of sugarcane to toxin from *H. sacchari*. We have now developed the electrolyte leakage assay for use in isolation of toxin and for comparison of sugarcane clones for sensititivity to toxin.

Data indicate that toxins from several other plant pathogenic fungi are significant determinants of pathogenicity and host-selectivity (10). Toxin from *H. sacchari* has not been evaluated critically for an overall role in development of the disease in sugarcane. However, a study of the action of helminthosporoside (a toxic compound from *H. sacchari*) (14) often is cited as the most definitive work to date on the molecular basis of disease development and disease resistance in plants. A primary objective of this study was to re-examine the basic role of the toxin. No final conclusions are drawn, but the data indicate a more complex situation than was expected. It is possible that the toxin is a disease determinant for some clones or genotypes, not for others.

MATERIALS AND METHODS

The following clones of sugarcane were obtained from J. L. Dean of the USDA Research Station at Canal Point, FL: CP29-320; CP33-229; 51NG-127; 57NG-100; CP63-588; CP44-101; F31-962; Hinahina; Co 453 and CP57-603. Clones H50-7209, H52-4610, and 51NG-97 were obtained from G. A. Strobel of Montana State University. Other clones were obtained from K. E. Damann, Jr., of Louisiana State University. The plants were grown during all seasons (1977-1979) in 5 gal plastic pots in the greenhouse, without supplemental lights, at temperatures above 18 C. Young, but fully-expanded, leaves were used for assays.

Isolates of *Helminthosporium sacchari* were obtained from J. C. Comstock (Hawaii Sugar Planters' Assoc.), G. A. Strobel, and J. L. Dean. The isolates were similar in ability to produce toxin. Stock cultures were maintained on cane leaf agar (15). Cultures for toxin production were grown for 21 days at 21–23 C, in still culture, in 1-L Roux bottles each containing 200 ml of modified Fries medium supplemented with 0.1% yeast extract (9). Cultures were harvested by filtration through cheesecloth, followed by filtration through Whatman No. 1 paper.

The procedures for isolating toxin will be described fully in another paper (Livingston and Scheffer, *unpublished*). Major sequential steps in the procedure were as follows: adsorption of toxin from concentrated culture filtrates to charcoal (Norit A), followed by rinses with dilute ethanol solutions and elution of toxin with 1% ammonium hydroxide in 50% ethanol; chromatography on an LH-20 Sephadex column developed with 50% methanol; QAE Sephadex anion exchange chromatography; thin-layer chromatography on Merck SG-60 plates developed with acetone: water (9:1); chromatography on a compound column containing layers of G-25 Sephadex, SP Sephadex cation exchange resin, and QAE Sephadex anion exchange resin; chromatography on a Bio-Gel P-2 column (115 × 1.35 cm). The trimethylsilyl derivative of the final preparation gave a single definite peak with gas chromatography, and a single spot on each thin-layer chromatogram developed and visualized with several different solvents and indicators. The preparation caused leakage of electrolytes from leaves of sugarcane clone Co 453 at 10 ng/ml. Toxin did not lose activity when stored 2 mo in methanol at −15 C.

Sugarcane leaves for the standard toxin assay were chosen from the youngest fully-expanded leaves at the top of the shoot. At least four leaves from four different plants were harvested for each assay. Leaf disks (1 cm in diameter) were cut, rinsed, and random samples were placed in scintillation vials. Each vial contained eight disks (~0.1g) in 2 ml of toxin solution or water. Vials were incubated on a shaker (22 C, 120 strokes per minute) for 1 hr. Toxin solutions or water were then removed, disks were rinsed for 10 min with several changes of distilled water, and 5 ml of water was added as a leaching

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solution. The vials were incubated on a shaker, and conductivity (μmhos, or μSiemen's units) was taken at 0.5, 1, 2, and 3 hr with a pipette-type electrode coupled with a Markson Electromark conductivity meter. All assays and all experiments were repeated two or more times.

RESULTS

Evaluation of assays involving visible symptoms of toxicity. The assay used by Steiner et al (11,13) was tested for clonal comparisons. Excised leaf sections (15 cm long) of several different clones (Co 453, CP52-68 CP61-37, H50-7209, and others which are susceptible, intermediate, and resistant to the fungus) were placed on moist paper in porcelain pans. A droplet (1 or 5 μl) of toxin-containing solution or water was placed on a fine puncture wound near the basal end of each leaf; the pans were then covered with transparent plastic films and incubated at room temperature (21–23 C) in either diffuse light or darkness. Toxin was used in a series of concentrations. The toxic effects described by others (13) were obtained, except that we did not observe recovery from initial water congestion in tissues (14). Susceptible, toxin-treated leaves showed water congestion within 12 hr, "runner lesions" by 24 hr and maximum effects by 48 hr after exposure. The time sequences and severity varied with toxin concentration. However, the results were erratic; some leaves of susceptible clones failed to respond and some leaves from relatively resistant clones gave symptoms after exposure to toxin. The assay was tested a number of times, with careful attention to details; the results always lacked precision. We concluded that the assay is not suitable for differentiating sugarcane clones with intermediate levels of resistance.

We also tested a possible assay in which basal ends of excised leaves were placed in small vials of water or partially purified toxin solutions. A series of toxin dilutions was used and the cuttings were incubated under fluorescent lights at 21–23 C. Within 12 hr, susceptible tissues developed water congestion, which was followed by necrosis, drying, and cessation of water uptake. Leaves of resistant clones had no early symptoms and continued to take up the solutions; thus, resistant leaves took in more toxin than did susceptible leaves, and eventually showed some damage. The method can be considered a valid assay only if each leaf is allowed to take in a measured amount of toxin solution.

Toxin-induced loss of electrolytes. Some preliminary information on the pattern of toxin-induced leakage was needed for development of a reliable assay. Leaves from the tops of young plants (3 mo old) responded about the same as did comparable leaves from older plants (8 mo). All plants from a given clone gave approximately the same response. Leaves from one clone (CP47-193) changed in relative sensitivity to toxin after cuttings were excised and held at room temperature for several days; other clones did not change in sensitivity after similar excision and aging. All subsequent clonal comparisons were made with freshly-harvested leaves. Vacuum-infiltration with toxin solutions was not necessary; infiltrated leaves had no more than a slight increase in early losses of electrolytes, compared to leaves that were not vacuum infiltrated. Maximum leakage for a given concentration of toxin was obtained when leaf disks were exposed to toxin for 1 hr, rinsed for 10 min, and placed in water for determination of subsequent leakage.

Changes in rates of leakage following exposure to toxin were determined by continuous monitoring, with a recorder connected to the conductivity meter. Twenty leaf disks from a toxin-sensitive clone were placed in 12.5 ml of toxin solution which was stirred continuously and circulated through a tubular electrode. Each of three such experiments showed increased electrolyte leakage within 15 min of exposure to toxin (Fig. 1). The leakage rate continued to increase for 2–3 hr; thereafter, it diminished (Fig. 2). Leaf disks that were left for 18 hr in the highly purified toxin solution (Fig. 2) lost the same amounts of electrolytes, and had the same rate changes, as did disks that were exposed to toxin only during the 1st hr (provided the losses into the rinse water were considered) (Fig. 1). Results of these experiments indicated the proper exposure times and the best times for termination of assays.

The effects of toxin concentration on leakage of electrolytes was tested. Standard assay procedure, the most susceptible clone (Co 453) and highly purified preparations of toxin were used. Results (Fig. 3) showed that electrolyte losses increased linearly with increases in toxin concentration from 0.1 to 0.5 μg/ml. This highly purified toxin preparation gave maximum effects (saturation) at 50

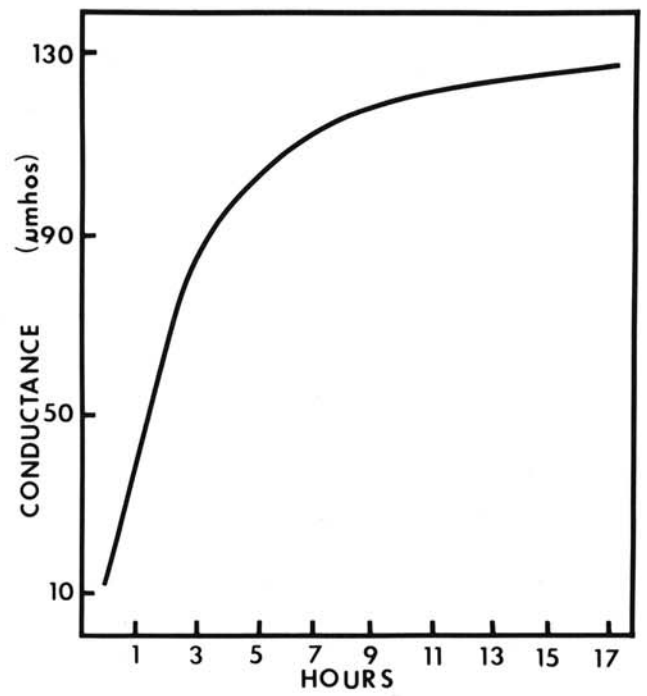

Fig. 1. Toxin-induced loss of electrolytes from leaf disks of sugarcane clone Co 453, as determined by continuous monitoring. Tissue was exposed to the toxin preparation (50 μg/ml) for 1 hr, rinsed, and placed in water at 0-time. Toxin was from a QAE Sephadex column.

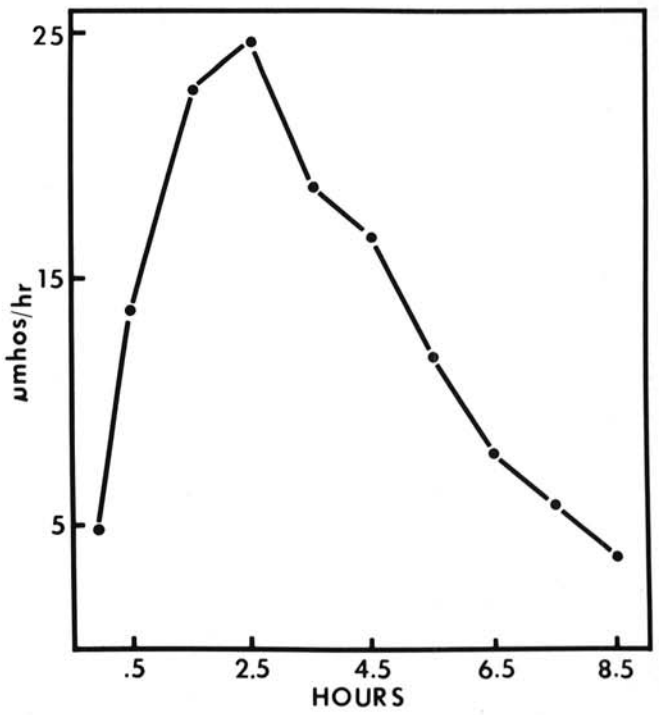

Fig. 2. Rates of electrolyte leakage induced by toxin from sugarcane leaf disks (clone Co 453). Tissue was exposed to toxin (50 μg/ml) during the entire experiment, and leakage is expressed as μmhos/hr. Toxin was from a QAE Sephadex column.

TABLE 1. Minimum concentrations of *Helminthosporium sacchari* toxin required to induce leakage of electrolytes from leaf disks from selected sugarcane clones[a]

Clone	Relative sensitivity	Toxin concentration (μg/ml)	Results
Co 453	Highly sensitive	0.01	Leakage
CP52-68	Sensitive	0.15	Leakage
CP63-588	Insensitive	10.0	Leakage
H52-4610	Highly insensitive	100.0	No leakage

[a]Leaf disks were exposed to toxin for 1 hr, washed, and placed in the leaching solutions. Electrolyte losses during 3 hr were determined. The toxin was in a highly purified preparation from a Bio-Gel P-2 column.

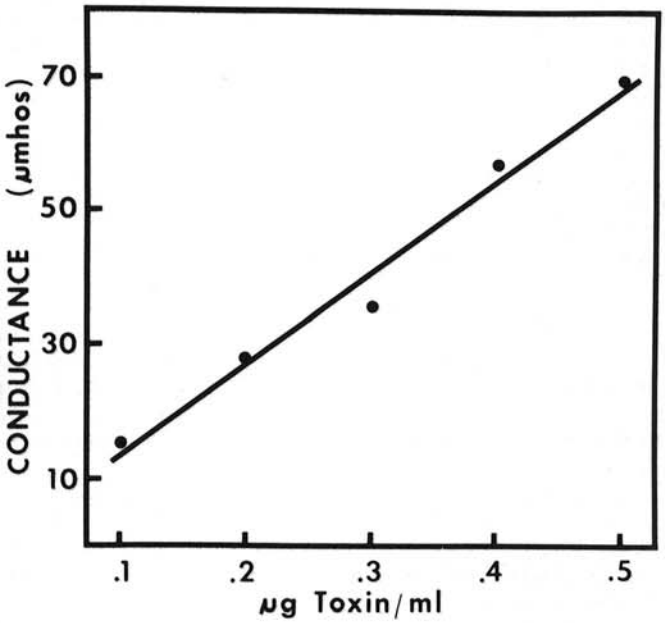

Fig. 3. Effect of toxin concentration on leakage of electrolytes from sugarcane leaf disks (clone Co 453). Tissue was exposed to highly purified toxin (from a Bio-Gel P-2 column) for 1 hr, rinsed, and placed in water at 0-time. The values are conductivity at hour 3 minus conductivity at hour 0.5

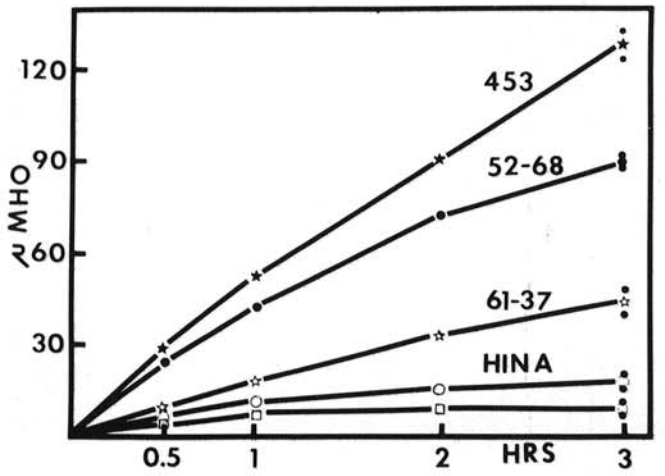

Fig. 4. Toxin-induced leakage of electrolytes from selected clones of sugarcane. Leaf disks were exposed to toxin for 1 hr, rinsed, and placed in water at 0-time. Conductivity values for control leaves without toxin (average = 7.7 at hour 2) were substracted. Toxin, from a QAE Sephadex column, was used at 25 μg/ml. Standard deviation is indicated for hour 3. Clones were Co 453 (★), CP52-68 (●), CP61-37 (☆), Hinahina (○), and CP63-588 (□).

μg/ml, causing all electrolytes to be lost from the leaves in 28 hr. Nonsaturating concentrations failed to cause complete leakage; instead, the loss curve flattened. Total electrolytes in tissues were determined after boiling, or after freezing and thawing several times; these treatments caused conductivities of ambient solutions to reach 300–350 μmhos in tests utilizing the same volumes and tissue weights as used in the assays. Overall, the data indicate that assays based on leakage of electrolytes can be relatively quantitative, provided the same conditions are always used and the proper standards are included.

Comparative sensitivity of sugarcane clones. The lowest concentrations of the highly purified toxin that were required to cause loss of electrolytes from sensitive, intermediate, and highly insensitive clones were determined by standard assay procedures. Toxin at 0.01 μg/ml caused leakage from leaf disks of clone Co 453, whereas 100 μg/ml had no effect on clone H52-4610 (Table 1). The values for the intermediate clones fell between these extremes. Thus, the most insensitive clone tolerated at least 10,000 times higher concentration of toxin than did the highly sensitive clone. To conserve highly purified toxin, higher concentrations were not used with the insensitive clone. Toxin concentrations lower than those indicated in Table 1 were observed on several occasions to cause an uptake of electrolytes by tissues of clones Co 453 and CP52-68. A similar, but more pronounced, phenomenon was caused by the host-selective toxin of *H. carbonum* (16).

Seventeen different clones of sugarcane that vary in susceptibility and resistance to *H. sacchari* were tested during all seasons for relative sensitivity to toxin. Toxin-sensitive (Co 453) and toxin-insensitive (H50-7209 or H52-4610) clones were included as standards in each test. The standard assay procedures and several different toxin preparations were used. The toxin used in the November tests (see Table 2) was prepared by Norit adsorption followed by chromatography on a Bio-Gel P-2 column. This preparation was used at 1.0 μg/ml (which induced electrolyte losses only in the most sensitive clones) and at 50 μg/ml. Toxin used in the

TABLE 2. Relative sensitivity of sugarcane clones to toxin from *Helminthosporium sacchari*, expressed as a percent of the electrolyte losses induced in susceptible clone Co 453

Clone	Relative electrolyte losses (% Co 453)		Reaction to fungus[e]
	April[a,b]	November[a,c]	
H50-7209	0	0	4
H52-4610	0	0	–
CP63-588	4.5	3	R
57NG-100	4	15	–
L60-25	8	6	–
F31-962	12	11	–
CP57-603	12	11	6
CP47-193	24	10	–
Hinahina[d]	13	54	S
51NG-127	38	34	–
CP44-101	46	47	3
CP33-229	46	61	1
CP61-37[d]	32	75	–
CP52-68	75	70	6
CP 29-320	108	86	1
Co 453	100	100	9
51NG-97	112	105	–

[a]All clones were retested in December, and some clones were retested in May. Ratings were essentially as given here.

[b]Toxin, an eluate from a QAE Sephadex column, induced electrolyte leakage from leaf disks of clone Co 453 at 0.01 μg/ml. Toxin was used at 25 μg/ml. Representative clones were tested with highly purified toxin (from a Bio-Gel P-2 column), with relative results that agreed with those reported here.

[c]Toxin (used at 50 μg/ml) was prepared by Norit absorption followed by chromatography on Bio-Gel P-2.

[d]Sensitivities of these clones were considered to vary with the season.

[e]Numbers refer to cane disease index ratings on a 1 (resistant) to 9 (susceptible) scale. Letters refer to J. L. Dean's ratings (R = resistant or S = susceptible; *personal correspondence*, 1979).

April tests was more highly purified; it came from a QAE Sephadex column. The preparation caused electrolyte losses from clone Co 453 at 0.1 μg/ml, and was used at 5 and 25 μg/ml. Relative sensitivities of the clones were the same at the low and at the high concentration of toxin, but losses from all sensitive clones were greater at the higher concentration. The results were confirmed by use of highly purified toxin preparations, in limited tests with a few representative clones.

The 17 clones varied in reaction to toxin, from highly insensitive (H50-7209 and H52-4610) to highly sensitive (Co 453 and 51NG-97) (Table 2). At least two intermediate levels of tolerance are evident. Results for most of the clones were consistent from season to season; the exceptions were clones CP61-37 and Hinahina, which varied with the season. The data in Table 2 are expressed as percent of losses by the most sensitive clone; data showing changes in absolute conductivity values with time, for selected clones, are shown in Fig. 4.

Some of the clones were used in other tests throughout the year, including mid-summer, when high temperatures in the greenhouse caused decreases in sensitivity of tissues to toxin (2). However, the relative sensitivity ratings agreed with those reported in Table 2. In two midsummer experiments, whole plants of representative clones were held at 21–23 C for 3 days before the leaves were used in assays. Again, the relative sensitivities to toxin were in agreement with the values reported in Table 2.

Two clones (CP61-37 and Hinahina) varied with season in response to toxin (Table 2, Fig. 4). Both clones were relatively resistant during the spring and summer months, even when plants were held at 21–23 C for 3 days prior to the tests. However, leaves produced during the short-day, low light conditions of late fall and early winter were much more sensitive to toxin. This change in tolerance of clone CP61-37 was confirmed many times in tests throughout the year. There were confirmatory assays on clone Hinahina, in addition to the data shown in Table 2 and Fig. 4.

We examined the possibility that our preparations contained more than one toxin from *H. sacchari,* because this might affect relative sensitivity of clones. Concentrated culture filtrates were precipitated with methanol and extracted with chloroform; toxin in the aqueous phase was then partitioned into butanol. Butanol was removed and aqueous samples were chromatographed on Sephadex G-15 in 18-, 25-, and 45-cm columns. Two-milliliter fractions from the columns were collected and assayed by the electrolyte leakage method. Contrary to the reports of others (11,13) there was only one peak of toxic activity. Thin-layer and paper chromatography of toxin preparations at various stages in processing also showed only one active form of the toxin.

DISCUSSION

The validity of this and other work on toxin from *H. sacchari* depends upon reliable assays. An assay based on induction of visible symptoms in leaves by small droplets of toxin solutions (13) was found to lack the precision needed for our purposes. However, results of this assay were in general agreement with results of an assay based on toxin-induced loss of electrolytes. An assay based upon transpiring cuttings that take up toxin solutions also was unsatisfactory. To date the most reliable assay that has been used is based on toxin-induced leakage of electrolytes from leaf tissue. The procedure requires use of replicate samples, standardized procedures, and resistant tissue controls. The assay can be quantitative, as shown by data obtained with a series of concentrations of both crude and highly purified toxin preparations. The method was suitable for guiding the isolation of toxin and for comparison of relative sensitivities of sugarcane clones. For clonal comparisons, each assay must include standards consisting of a highly resistant and a highly sensitive clone.

Toxin preparations with several different levels of purity were used in these experiments. However, confirmatory experiments with highly purified toxin were included. Evidence of high purity was a single spot obtained on thin-layer chromatograms developed and visualized with several different solvent systems and indicators. The best of those preparations, analyzed by gas chromatography, gave a single peak. Characterization of the toxin by mass spectroscopy, nuclear magnetic resonance, and other instrumental methods will be reported in a later paper.

Other workers have reported evidence for at least two different toxins from *H. sacchari* (11,13). Obviously, sugarcane clones could differ in reaction to different toxins, or even to different forms of the toxin. Therefore, we re-examined the evidence for different toxins, using several isolates of the fungus (including one from G. Strobel). The results were negative; using column- and thin-layer chromatography, we found no evidence of more than one form of toxin. Thus, we tentatively ruled out different toxins as factors in our comparisons. It is still possible that other strains of the fungus might yield other forms of toxin. However, the work of Steiner and Byther (12) indicated that cultures of *H. sacchari* from Australia, Florida, and Hawaii had comparable toxic effects, and probably produced the same or at least a closely related form of the toxin.

The major objective of this work was to compare clones of sugarcane for relative sensitivity to toxin from *H. sacchari.* We tested the clones during all seasons and under various experimental conditions. The results showed wide differences in reaction to toxin, from highly sensitive through intermediate levels to highly insensitive. The most insensitive clones tolerated at least 10,000 times higher concentrations of toxin than did the most sensitive clones. There were at least two (perhaps more) intermediate levels of clonal reaction. With two exceptions, the relative ratings of clones were consistent throughout the year. Clone CP61–37 and Hinahina were less sensitive to toxin during the spring and summer than during the late fall and early winter; there appears to be more involved in this effect than simple heat-induced tolerance (2). The basis of differences in clonal sensitivity is not known; perhaps several genes control resistance to toxin. Perhaps differences in sensitivity are related to differences in ploidy and chromosome numbers in sugarcane, a variable species in this respect (6).

The major reason for rating the clones was to compare host sensitivity to toxin with relative susceptibility to the fungus. The comparison proved to be more difficult than anticipated, because reliable data have not been published for many of the clones, and because inoculations in the greenhouse may not give satisfactory data. Nevertheless, enough information is available to see that reaction of some clones to toxin is not correlated with reaction to the fungus in the field. Even though clone CP29-320 is listed in the Cane Index (3) as highly resistant to the fungus, we found it to be among the more toxin-sensitive clones. Clone H50-7209 is intermediate in susceptibility to the fungus (3,8), with a rating of 4 on a 1 (resistant) to 9 (susceptible) scale (7), yet it is highly resistant to the toxin. Clone CP52-68 rates 6 on the 1 to 9 scale and is relatively sensitive to the toxin, whereas clone CP57-603 also rates 6 and is relatively insensitive to toxin. Outbreaks of disease caused by *H. sacchari* have been reported in Florida on clone CP57-603 (5). Clone Hinahina is very susceptible to the fungus in Florida (J. L. Dean, *personal communication*), yet it is intermediate or tolerant to the toxin (Table 2).

The data suggest that host-selective toxin from *H. sacchari* may be only one of several factors involved in host-specificity by the fungus. The toxin may determine pathogenicity to some clones, but not to others. Mechanisms of pathogenicity that do not depend on toxin production may be involved in the interaction of the pathogen with some clones. On the part of the host, there may be mechanisms in control of susceptibility and resistance that are not related to toxin reaction. These possibilities must be clarified before the toxin can be considered the major determinant of disease induced in sugarcane by *H. sacchari.* The data also imply that toxin should be employed with caution in screening for disease resistance.

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