Characterization of Sugarcane Response to *Bipolaris sacchari*: Inoculations and Host-Specific HS-Toxin

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ABSTRACT

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Twenty-seven sugarcane clones inoculated with *Bipolaris sacchari,* causal agent of eyespot disease, were classified as susceptible or resistant based on the presence or absence of distinct chlorotic halos surrounding lesions on leaves. Under the inoculation conditions used in this study, all lesions on resistant plants were smaller than 3 mm; the proportion of lesions larger than 3 mm on susceptible clones ranged from 0.2 to 82.2%. The total number of lesions was not related to resistance or susceptibility. All clones classified as resistant to *B. sacchari* were insensitive to a host-specific toxin (HS-toxin) produced by the pathogen, whereas all susceptible plants produced a water-soaked streak when 1 μl of partially purified HS-toxin was placed in a hole punched in a leaf segment. These results indicated that sensitivity to HS-toxin was an important factor in determining whether the pathogen could cause disease symptoms on the plant. Measurement of toxin-induced electrolyte leakage was not as reliable for differentiating between resistant and susceptible clones. In most susceptible individuals, the degree of disease severity was correlated with the level of sensitivity to HS-toxin; however, there were three clear exceptions, suggesting that in some clones, other factors besides sensitivity to HS-toxin might be involved in determining the extent of symptom expression.

Eyespot disease of sugarcane (*Saccharum* spp.), caused by the fungal pathogen *Bipolaris sacchari* (E.J. Butler) Shoemaker (formerly *Helminthosporium sacchari* E.J. Butler in E.J. Butler & Hafiz Khan), can be a devastating disease if susceptible cultivars are grown (5–7,11,14). The pathogen produces a host-specific toxin, HS-toxin. The level of sensitivity of sugarcane genotypes to HS-toxin has been measured by either a leaf-hole assay (15,16) or an electrolyte-leakage assay (2,10,15). Steiner and Byther (16) and Scheffer and Livingston (15) reported that clones sensitive to HS-toxin usually had a fairly high disease rating (i.e., they were more susceptible), whereas most toxin-insensitive clones had a low rating. However, in both cases, evaluation of inoculations was based on a subjective scoring system (8), and the severity of infection was not well quantified. In addition, clones that appeared to be resistant to the pathogen and sensitive to HS-toxin, and genotypes that were susceptible to the fungus and insensitive to toxin, were identified in both studies. Kang and Dean (9) categorized sugarcane responses to inoculations as high or low infection types based on the size of lesions and the presence or absence of halos; however, they did not analyze HS-toxin sensitivity. In this paper, we define criteria that can be used to clearly classify sugarcane plants as resistant or susceptible to *B. sacchari,* and we establish that response to HS-toxin is critical for determining whether eyespot symptoms of the disease will be expressed.

MATERIALS AND METHODS

Plant material. The sugarcane clones (*Saccharum* spp. hybrids) utilized in this study were chosen based on good pollen production so they could be selfed for a concurrent genetic analysis. Clones included: H72-1365, H73-6110, H73-7324, H74-4527, H75-3083, H76-5956, H76-8515, H77-0682, H78-0292, H78-1207, H78-2541, H78-4153, H78-6747, H78-7234, H79-2867, H79-5163, H79-6185, H80-2339, H80-3329, H80-4246, H81-2844, and H82-0032. Also included in this work were clones LA Purple (*Saccharum officinarum* L.) and Mol 5829 (*S. robustum* Brandes & Jesw. ex Grassl.) that were crossed for the genetic investigation. H109 and H65-7052 were used as susceptible and resistant controls, respectively, in all analyses; H78-0878 was used as an additional resistant control in spore inoculations.

Inoculations. *B. sacchari* spores were produced by a method similar that of to Comstock and Scheffer (3). Fungal mycelia (Hawaiian isolate ES11) were shake cultured at 200 rpm in Fries medium (12) supplemented with yeast extract at 1 g/L for approximately 7 days at 25 C. Mycelia were shredded in a Waring blender for 2 min and centrifuged for 10 min at 1,400 *g*, and the pellet was resuspended in 10 mM phosphate buffer (equal volumes of 15 mM NaH_2PO_4 and 5 mM Na_2HPO_4, pH 6.4). The suspension was centrifuged and resuspended in phosphate buffer three times before being poured into petri plates lined with filter paper; the plates were incubated in a growth chamber for 5–7 days with a 12 h, 24 C light/12 h, 20 C dark cycle. Prolific sporulation was noted after 2 days; however, higher spore concentrations were achieved if the plates were allowed to incubate for at least 5 days. Water was added to the plates, and the mycelia were separated from the spores by passing them through a 0.124-mm mesh sieve. This procedure retained most of the mycelia and allowed the spores to pass through. Spore concentrations were determined with a hemacytometer, and the suspension was diluted with water to 13,000 spores per milliliter.

The day before inoculation, potted sugarcane plants were placed in the growth chamber at 24 C to make them more receptive to infection (2). The spore suspension was sprayed onto the plants with an atomizer spray bottle, and the plants were covered with plastic bags for 2 days to maintain high humidity. Each plant

was evaluated twice for its response to the pathogen. Approximately 1 wk after inoculation, the number of lesions falling into three size categories (0–3 mm, 3–5 mm, and >5 mm) was estimated, and the presence or absence of distinct chlorotic halos surrounding the lesions was noted. After this evaluation, the plants were transferred to the greenhouse. Approximately 1 wk later, the plants were reevaluated to confirm the first evaluation. Occasionally, plants would exhibit more well-defined symptoms in the second evaluation, apparently due to more optimal plant growth conditions in the greenhouse. All clones were inoculated three times (one replicate per inoculation); resistant and susceptible controls, as well as water-only treatments, were included.

HS-toxin preparation and purification. HS-toxin was prepared according to Steiner and Byther (16), and partially purified by the protocol of Macko et al (13). Mycelia of *B. sacchari* (Hawaiian isolate ES11) were grown for 21 days at 24 C on supplemented Fries medium without shaking. The culture fluid (approximately 6 L) was separated from mycelia by passing it through eight layers of cheesecloth and one layer of Miracloth (Calbiochem-Behring, La Jolla, CA), centrifuging for 15 min at 40,000 *g*, and filtering through a 0.45-μm nylon membrane (Schleicher & Schuell, Keene, NH). The fluid was then passed through a 2.5 × 60 cm high-performance liquid chromatography (HPLC) column of reverse-phase C_{18} packing (55–105 μm) (Waters Associates, Milford, MA) at 10 ml/min, followed by a 30-min water wash. HS-toxin was eluted with a 1,600-ml linear gradient of 0–100% water-acetonitrile (Mallinckrodt, Inc., Paris, KY) at 10 ml/min. Fractions (12 ml) were tested for host-selective activity with the leaf-hole assay (described in next section), including sensitive and insensitive controls (H109 and H65-7052, respectively). Those fractions producing water-soaked streaks only on the sensitive genotype were pooled, and the acetonitrile was evaporated. The total volume of the pooled fractions ranged from 60 to 70 ml. The HS-toxin levels of pooled fractions from each toxin preparation were quantified according to bioactivity and actual toxin content.

For the bioactivity quantification, a dilution series (undiluted to 1/10,000 with 10× increments) was prepared, and each concentration was tested for toxin-induced electrolyte leakage (described below) on the sensitive control (H109), with three replicates per test. The concentration of HS-toxin in each preparation was determined by injecting 2 μl of sample into an analytical HPLC column (Phenomenex C_{18}, Phenomenex, Inc., Torrance, CA). The toxin was eluted with 19% acetonitrile (in water) at 1 ml/min, and fractions were monitored at 203 nm. The toxin concentration of each preparation was calculated by comparing the area under the peak corresponding to toxin with that of a 1-μg standard. HS-toxin isomer C, the largest component of each preparation, was the only form of toxin quantified by this procedure, since it accounts for most of the toxicity in HS-toxin preparations (4). Smaller quantities of isomers A and B, as well as a few unidentified compounds, were also present in each preparation.

Leaf-hole assay. The leaf-hole assay procedure has been described (16). The youngest fully expanded leaf blades were collected from field-grown plants 2 days before the assay and placed in the growth chamber with their bases immersed in water. On the day of the assay, 18-cm segments were cut from the middle portion of the leaf blades, unless otherwise noted. Four small holes were punched near the base of the segments (two on each side of the midrib) with a needle (18G11/2, Sigma Chemical Co., St. Louis, MO). Undiluted HS-toxin (1 μl, approximately 600

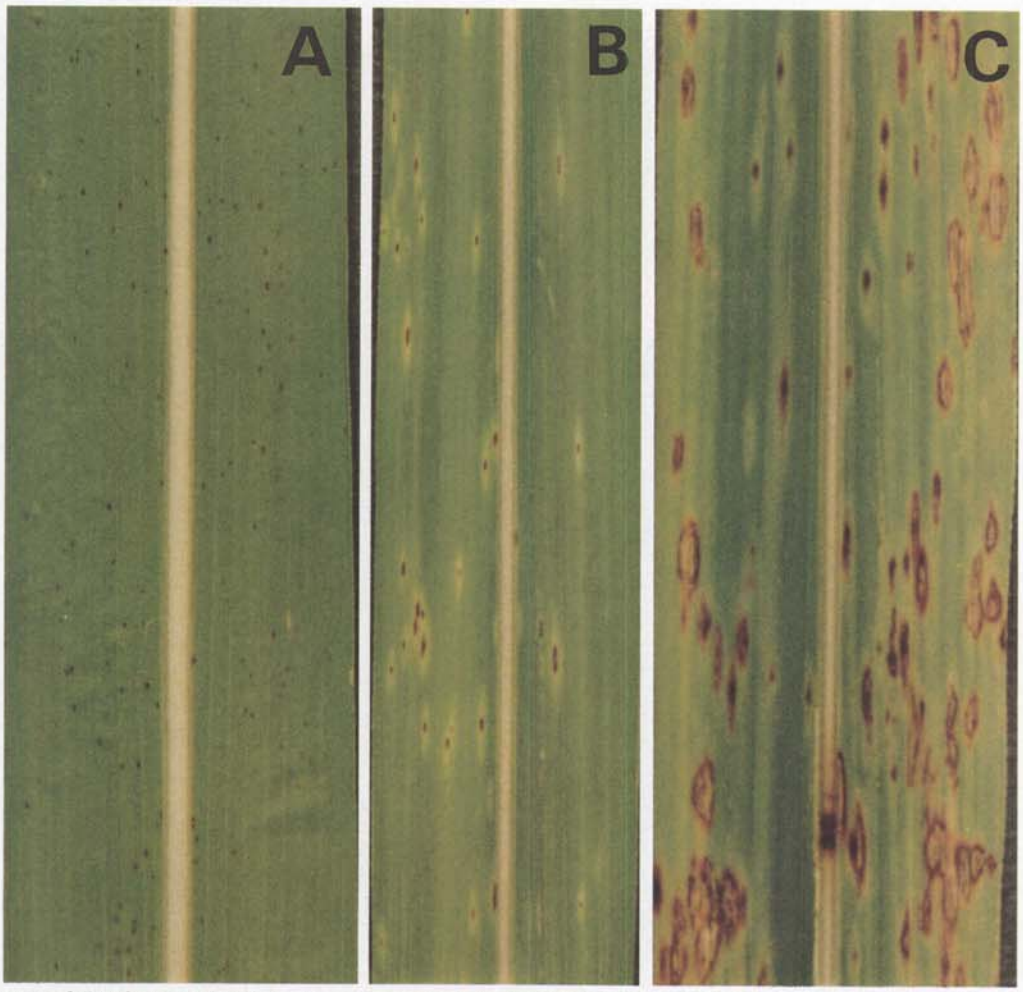

Fig. 1. Typical symptoms of *Bipolaris sacchari* infection on sugarcane. **A,** Resistant reaction. Lesions smaller than 3 mm with no halos surrounding them. **B,** Low susceptibility reaction. Most lesions less than 3 mm but with distinct chlorotic halos. **C,** High susceptibility reaction. Most lesions larger than 3 mm with very distinct halos. All clones designated as resistant had a disease response similar to A; disease response ranged between B and C among the clones classified as susceptible.

ng) was placed in two of the holes on opposite sides of the midrib, and 1 μl of water was deposited in the other two holes. The leaf segments were placed upright in an enclosed glass vessel walled with chromatography paper moistened with water and incubated in the growth chamber. After 48 h, the leaf segments were placed in front of a black background, and the length of the water-soaked streaks originating from the holes were measured.

Electrolyte-leakage assay. Electrolyte-leakage assays were performed using the protocol of Scheffer and Livingston (15). Leaves were collected and pretreated the same as in the leaf-hole assay. For each clone, several disks (1.1 cm diameter) were punched from the middle section of the leaf blade (excluding the midrib), and a random sample of eight disks was incubated in 3 ml of diluted HS-toxin for 1.5 h. The concentration of toxin that induced 50% leakage after 24 h was extrapolated from the bioactivity dilution series (described previously) for each preparation. This concentration (approximately 60 μg/ml) was used to determine the level of sensitivity of the clones. A 2.5-fold dilution of this concentration (approximately 25 μg/ml) was used to assay leaf tissue ages with the susceptible control (H109). Two preparations were used in this study, one in the clone analysis and another in the tissue-age investigation. The disks were then thoroughly rinsed with water and placed in 10 ml of double-distilled water. Solution conductance was measured with a conductivity bridge (Model 34, Yellow Springs Instrument Co., Yellow Springs, OH) 6 and 24 h after placing the disks in water. Measurements were also taken on double-distilled water lacking disks and on 1 M NaCl to calibrate the meter. To determine the total quantity of electrolytes in each sample, the samples were autoclaved and incubated for at least 24 h, and the conductance of the solution was measured. Susceptible and resistant controls were included in all assays, and disks treated with water instead of HS-toxin were also included for each treatment or clone. Percent electrolyte leakage was calculated as: $(L_{Tox}/L_{Tottox}) - (L_{Wat}/L_{Totwat})$, where L_{Tox} = toxin-induced electrolyte leakage, L_{Tottox} = total electrolytes in the toxin treated samples, L_{Wat} = electrolyte leakage in water controls, and L_{Totwat} = total electrolytes in water controls. All statistical analyses were performed on a personal computer using the Statistical Analysis System (SAS Institute, Inc., Cary, NC).

RESULTS

Response of sugarcane clones to *B. sacchari* spore inoculations. Twenty-seven sugarcane clones were inoculated three times each with *B. sacchari* spores. Figure 1 shows typical symptoms. Two distinct responses to the pathogen were observed among the clones. Thirteen of the clones had only small lesions (<3 mm) with no distinct chlorotic halos (Fig. 1A, Table 1). The other 14 clones had lesions that were frequently larger than 3 mm with distinct halos (Fig. 1B and C, Table 1). Halos were most prominent on the basal portion of the youngest fully expanded leaf. They were usually not as prominent, or were absent, on older leaves and leaf tips. The proportion of large lesions (>3 mm) varied

TABLE 1. Response of sugarcane clones to *Bipolaris sacchari* inoculations and host-specific HS-toxin produced by the pathogen

	Inoculations[y]				HS-toxin assays[z]	
Clone	Lesions > 3 mm (%)	Total lesions (no.)	Halos	Response to pathogen	Leaf hole streak length (cm)	Electrolyte leakage (%)
H109	82.2 a	1,308.3 ab	+	S	16.5 a	24.9 b
H78-6747	41.2 b	1,458.3 ab	+	S	14.9 a	34.2 a
H80-4246	28.7 bc	776.7 ab	+	S	1.4 d	2.2 ef
LA Purple	23.6 cd	537.5 ab	+	S	2.0 d	2.8 ef
H81-2844	20.1 cde	485.0 ab	+	S	4.8 c	7.9 d
H80-2339	14.8 cdef	1,950.0 a	+	S	1.1 d	3.7 ef
H78-7234	14.5 cdef	465.0 ab	+	S	1.1 d	3.1 ef
H78-2541	11.5 cdef	191.7 b	+	S	1.0 d	0.6 f
H74-4527	8.1 def	490.0 ab	+	S	0.7 d	3.9 ef
H78-4153	7.2 def	666.7 ab	+	S	0.3 d	1.9 ef
H76-5956	5.3 def	368.3 b	+	S	1.3 d	13.3 c
H78-0292	4.2 ef	293.3 b	+	S	8.8 b	14.6 c
H79-5163	3.9 ef	871.7 ab	+	S	2.6 cd	4.9 de
H78-1207	0.2 f	693.3 ab	+	S	0.2 d	1.2 ef
H65-7052	0.0 f	91.7 b	−	R	0.0 d	0.3 f
H72-1365	0.0 f	241.7 b	−	R	0.0 d	0.5 f
H73-6110	0.0 f	600.0 ab	−	R	0.0 d	0.2 f
H73-7324	0.0 f	158.3 b	−	R	0.0 d	0.3 f
H75-3083	0.0 f	600.0 ab	−	R	0.0 d	0.4 f
H76-8515	0.0 f	450.0 ab	−	R	0.0 d	0.7 f
H77-0682	0.0 f	408.3 b	−	R	0.0 d	0.0 f
H78-0878	0.0 f	533.3 ab	−	R	0.0 d	0.4 f
H79-2867	0.0 f	358.3 b	−	R	0.0 d	0.1 f
H79-6185	0.0 f	216.7 b	−	R	0.0 d	0.0 f
H80-3329	0.0 f	150.0 b	−	R	0.0 d	0.1 f
H82-0032	0.0 f	866.7 ab	−	R	0.0 d	0.6 f
Mol 5829	0.0 f	375.0 b	−	R	0.0 d	0.6 f

[y] Clones were inoculated three times (one replicate per inoculation); the total number of lesions and the proportion of lesions larger than 3 mm was estimated, and whether or not distinct halos surrounded the lesions was noted (+ = yes, − = no). The presence or absence of halos was the main criterion used to determine the response of the plants to the pathogen (S = susceptible, R = resistant). Significant differences among clones were tested by analysis of variance with Duncan's multiple range test (least significant difference values: Percent lesions > 3 mm = 20.0% and total number of lesions = 1,566, $\alpha = 0.05$). Variance among replicates was significant for the total number of lesions measurement but not for percent lesions > 3 mm, even when only susceptible clones were considered ($\alpha = 0.05$).

[z] Two assays were used to measure level of sensitivity of clones to HS-toxin produced by *B. sacchari*, with four replicates per assay. Leaf hole streak length was a measure of the length of the water-soaked streak extending from the HS-toxin application hole upward in the leaf-hole assay. Electrolyte-leakage numbers represent the portion of the total cellular electrolytes that leaked from leaf disks treated with HS-toxin (6-h reading). All values were corrected for leakage of controls incubated in the absence of toxin. For both assays, clones were tested for significant differences by analysis of variance with Duncan's multiple range test (least significant difference values: leaf hole lesion length = 3.0 cm and electrolyte leakage = 4.2%, $\alpha = 0.05$). Variance among replicates was not significant for either toxin assay even when only susceptible individuals were considered ($\alpha = 0.05$).

significantly among clones that produced halos (Table 1). However, total number of lesions was not significantly different among most clones (Table 1).

Sugarcane sensitivity to HS-toxin compared with response to spore inoculations. The leaf-hole and electrolyte-leakage assays were used to quantify the sensitivity of the 27 sugarcane clones to HS-toxin. In the electrolyte-leakage assay, the 6- and 24-h readings were highly correlated according to the Pearson product moment coefficient of correlation ($r = 0.98$, $P = 0.0001$). The 6-h reading was used in Table 1. Results of the two toxin assays were also strongly correlated ($r = 0.94$, $P = 0.0001$).

In the leaf-hole assay, all clones that produced halos in response to the pathogen formed a water-soaked streak when treated with HS-toxin, whereas none of the individuals lacking halos developed a detectable streak; however, in most cases (10 of 14), the streak was not considered significantly longer than 0.0 cm (Table 1). In the electrolyte-leakage assay, all genotypes that did not exhibit halos had a low toxin-induced loss of electrolytes (Table 1). However, most of the halo-producing clones (8 of 14) also had electrolyte-leakage levels that were not significantly higher than the insensitive control, H65-7052 (Table 1).

To determine whether the level of sensitivity to HS-toxin was related to the severity of pathogen infection, we calculated correlation coefficients comparing the percentage of lesions larger than 3 mm from inoculations with measurements from the two toxin assays. A strong positive correlation between disease severity and level of toxin sensitivity was observed for both toxin assays (electrolyte leakage, $r = 0.74$, $P = 0.0001$; leaf hole, $r = 0.84$, $P = 0.0001$). However, two clones, H80-4246 and LA Purple, had a relatively low level of HS-toxin sensitivity in both assays but had severe responses to the pathogen; conversely, H78-0292 was highly sensitive to toxin in both assays but had a relatively low infection level (Table 1).

The relationship between sensitivity to HS-toxin and halo formation. As mentioned before, halos surrounding lesions were most prominent at the base of the youngest fully expanded leaf blade and were frequently absent on older leaves and leaf tips. To determine whether this observation was due to differences in sensitivity to HS-toxin because of varying tissue age, the leaf-hole and electrolyte-leakage assays were used on clone H109 to measure the effects of HS-toxin on the top, middle, and basal portions of the youngest leaf blade, and the middle portions of leaf numbers 1, 2, 3, 4, 5, 6, 8, and 10, starting with the youngest fully expanded leaf blade and moving basipetally to older leaves.

In both analyses, the 6- and 24-h readings in the electrolyte-leakage assay were strongly correlated (position on leaf blade, $r = 0.96$, $P = 0.0001$; leaf number, $r = 0.89$, $P = 0.0001$). Table 2 shows results from the 6-h reading. In the comparison of HS-toxin sensitivities among the three positions on the leaf blade, the results of the leaf hole and electrolyte leakage assays were not well correlated ($r = 0.56$, $P = 0.115$). No significant differences were noted in the leaf-hole assay; however, in the electrolyte-leakage assay, the base of the leaf blade was significantly less sensitive to HS-toxin than were the middle and tip (Table 2).

In the analysis of leaf position on the stalk, the two assays were significantly correlated ($r = 0.55$, $P = 0.005$), but much less so than in the analysis of the clones. In the leaf-hole assay, no significant differences were detected among the leaves; the only significant difference noted in the electrolyte-leakage assay was between leaf numbers 4 and 1 (Table 2).

DISCUSSION

Two distinct responses to *B. sacchari* inoculations were observed among 27 sugarcane clones: 13 clones had small lesions with no halos, and the other 14 had larger lesions with distinct chlorotic halos (Table 1). These two responses were similar to what was observed by Kang and Dean (9), who referred to them as low and high infection types. Those clones exhibiting the low infection type in response to the pathogen did not appear to sustain much tissue damage (Fig. 1A); however, clones that were a high infection type appeared to undergo significant necrosis, as evidenced by larger lesions and chlorosis within the halos (Fig. 1B and C). Therefore, we designated those genotypes that formed small lesions with no halos as resistant to the pathogen and those that formed larger lesions with halos as susceptible (Table 1).

Sensitivity to HS-toxin was measured with two assays: leaf-hole and electrolyte-leakage. Results of the two assays were strongly correlated in the analysis of the different clones. However, a weaker correlation was observed in the analysis of leaf age, and no significant correlation was detected in the study of sensitivities of different positions on the leaf blade. Most of the clones used in the clone study were relatively insensitive to HS-toxin according to both assays (Table 1), resulting in a high correlation between the assays. H109, the only clone used in the latter two studies, was very sensitive to toxin compared to the other clones (Table 1); this resulted in more variability among replicates (compare least significant difference values from Tables 1 and 2) and less correlation between the two assays.

From a statistical perspective, neither assay could be used to identify clones resistant or susceptible to *B. sacchari* (Table 1). However, the leaf-hole assay, using the presence or absence of a toxin-induced water-soaked streak as a criterion, without regard to streak length, successfully differentiated between the two phenotypes in all cases (Table 1). Therefore, our results indicated that all clones that were insensitive to HS-toxin were resistant to *B. sacchari,* whereas all plants sensitive to toxin, as evidenced by a water-soaked streak in the leaf-hole assay, were susceptible to the pathogen (Table 1). This indicated that, in the clones analyzed in this study, sensitivity to HS-toxin was a very important factor in determining whether the pathogen could cause eyespot symptoms. Correlations between sensitivity to HS-toxin and response to *B. sacchari* have been reported previously (15,16); however, in those studies, clones were identified that were insensitive to HS-toxin and susceptible to the pathogen, or sensitive to toxin and resistant to the pathogen. These exceptions may have been due to misinterpretation of their inoculation data. To evaluate inoculations, Steiner and Byther (16) used a subjective

TABLE 2. Effect of tissue age on sensitivity to HS-toxin produced by *Bipolaris sacchari*

Position on leaf blade or leaf number[y]	HS-toxin assays[z]	
	Leaf hole streak length (cm)	Electrolyte leakage (%)
Tip	11.4 a	28.8 a
Middle	7.4 a	25.0 a
Base	7.6 a	14.4 b
1	7.8 a	19.0 b
2	7.4 a	21.5 ab
3	10.7 a	20.5 ab
4	13.0 a	26.9 a
5	10.4 a	21.1 ab
6	10.2 a	26.1 ab
8	9.6 a	26.1 ab
10	7.6 a	19.7 ab

[y] Position on leaf blade indicates the section of the leaf blade sampled (base, middle, or tip); the youngest fully expanded leaf was used for both toxin assays. Leaf number indicates the position of the leaf on the stalk starting with the youngest fully expanded leaf blade and moving basipetally toward the older leaves; the middle portion of the leaf was sampled for both assays.

[z] Two assays were used to measure the level of sensitivity to HS-toxin. Leaf hole streak length was a measure of the length of the water-soaked streak extending from the HS-toxin application hole upward in the leaf-hole assay (six replicates). Electrolyte-leakage numbers represent the portion of the total cellular electrolytes that leaked from leaf disks treated with HS-toxin (6-h reading), with all values being corrected for leakage of controls incubated in the absence of toxin (three replicates). For both assays, leaf samples were tested for significant differences by analysis of variance with Duncan's multiple range test (least significant difference values: leaf hole streak length, position on leaf blade = 4.9 cm and leaf number = 6.4 cm; electrolyte leakage, position on leaf blade = 9.5% and leaf number = 7.8%, $\alpha = 0.05$). The position on leaf blade and leaf number studies were statistically analyzed separately.

numerical rating system, and Scheffer and Livingston (15) relied on the assessments of several previous investigators. In both cases, the inoculation data were not well quantified. We have observed occasional discrepancies between results from our inoculations and reported disease ratings (1). Moreover, Scheffer and Livingston (15) mainly used the electrolyte-leakage assay to measure the sensitivity of the clones to HS-toxin; in our study, eight of the 14 susceptible genotypes had electrolyte leakages that were not significantly greater than those observed for the resistant control, H65-7052. Therefore, it is not surprising that they identified clones that appeared not to respond similarly to HS-toxin and the pathogen. We cannot rule out the possibility that in some clones not analyzed in this study, factors other than HS-toxin sensitivity may have a role in determining whether or not symptoms of the disease will be expressed.

Scheffer and Livingston (15) reported that the leaf-hole assay was not reliable for differentiating clones with intermediate levels of resistance. Specifically, they reported that some clones considered resistant to the pathogen produced a streak, whereas susceptible clones occasionally failed to exhibit a streak. In our study, none of the resistant clones produced a water-soaked streak in the leaf-hole assay (Table 1). The discrepancy between the two results could have been due to misinterpretation of inoculation data in the previous study, since this data was supplied by previous investigators not involved in their study. Clones thought to be resistant may have actually exhibited a low level of susceptibility according to criteria from our analysis. Similar to the previous study, we had one clone, H78-1207, that failed to produce a streak in two of the four replicates in the leaf-hole assay (data not shown). This clone had low levels of susceptibility to both the pathogen and HS-toxin (Table 1). Toxin-induced tissue damage must be fairly extensive for a detectable streak to form. It was possible that damage had occurred in these replicates, but not enough to show visible symptoms.

In our analysis, the electrolyte-leakage assay could not be used to differentiate between resistant and susceptible clones. A possible reason for the greater reliability of the leaf-hole assay was that, in this assay, resistant plants and water controls showed no response to toxin; susceptible plants could easily be identified, even if they only produced a small streak. In the electrolyte-leakage assay, leaf disks from resistant clones and water controls lost some electrolytes due to wounding from cutting the disks; susceptible individuals could be identified only if they leaked significantly more than their water control. Even though the electrolyte-leakage assay was not a reliable qualitative indicator of resistance or susceptibility, it may be a better quantitative estimator of HS-toxin sensitivity in highly sensitive clones because of lower variability among replicates and ease of measurement (i.e., the streak in the leaf-hole assay was not always continuous, occasionally making it difficult to measure its length).

As mentioned before, all clones that were susceptible to *B. sacchari* also sustained some tissue damage in response to HS-toxin, as evidenced by toxin-induced water-soaking in the leaf-hole assay. In most cases, the degree of toxin-induced necrosis was positively correlated with the severity of symptoms in inoculations (Table 1). However, two clones had a relatively low sensitivity to HS-toxin but were very susceptible to the pathogen (H80-4246 and LA Purple), and one clone was very sensitive to toxin but had a low infection level (H78-0292) (Table 1). These three cases indicated that other factors besides sensitivity to HS-toxin might be involved in determining the extent of symptom expression. However, an additional possibility was that the impurities in the toxin preparation could have affected toxin sensitivity in these three clones.

The paucity of halos on leaf tips and older leaves could not be explained by low sensitivity to HS-toxin. In fact, according to the electrolyte-leakage assay, the lowest sensitivity to toxin was on the basal portion of the leaf blade (Table 2); this was the region where halos were most prominent in inoculations. We have three possible explanations for this observation. First, even though the most distinct halos were usually observed at the base of the leaf, the lesions in this region were frequently smaller than on the rest of the leaf. Since we observed a correlation between HS-toxin sensitivity and lesion length, it is possible that more prominent halos formed in this portion of the leaf because lesion extension was restricted by lower toxin sensitivity. Second, compared to the middle and tip of the leaf blade, the base is narrower and thicker, with vascular bundles that are closer together due to a decrease in air cavities (17). Either uptake of HS-toxin into the leaf disks or release of electrolytes may have been inhibited, thus resulting in a lower conductivity reading. Third, during inoculations, the spore suspension tended to roll down the leaves and accumulate at the base; this was especially the case on younger leaves due to their more upright growth. It is possible that a significantly greater number of spores could have been present at the leaf base during inoculations, resulting in more distinct halo formation in spite of lesser sensitivity to HS-toxin.

All sugarcane clones in this study were also inoculated with a second Hawaii isolate of *B. sacchari*. The two isolates appeared to have identical host ranges (data not published). It is possible, though, that other isolates may give different results from those reported here.

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