Partial Characterization and Use of a Host-Specific Toxin from *Helminthosporium sacchari* on Sugarcane

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ABSTRACT

Toxic filtrate was obtained from 20- to 24-day-old cultures of *Helminthosporium sacchari,* the causal agent of eye spot disease of sugarcane. Partial purification of the toxic compound was accomplished by treating concentrated filtrate with methanol (soluble), partitioning with chloroform (insoluble) and butanol (soluble), and fractionating by gel filtration (Sephadex G-15). The toxin was stable over extended periods of time and to heat of up to 144 C. Toxin production and fungus growth were affected by both temperature and time. Symptoms produced by the toxin and the fungus are similar. Only plants susceptible to the fungus are affected by the toxin. The toxin reaction of 182 sugarcane clones was significantly correlated ($r = .88$) to their reaction to the pathogen. Large-scale screening for resistance to eye spot disease can be accomplished accurately and rapidly by using this toxin. Phytopathology 61:691-695.

Eye spot disease, caused by *Helminthosporium sacchari* B. de Haan) Butl., is a widespread disease of sugarcane (*Saccharum* sp.) (6). Kruger named the disease eye spot (6) because early symptoms of the disease are elongated leaf lesions showing red centers surrounded by a narrow margin of chlorotic tissue. Shortly after the lesions are formed, reddish-brown streaks or runners initiate from the primary point of infection toward the tip of the leaf. A runner can be up to 3 ft long. The fungus can be isolated easily from the lesions but not from the runner area (6). Lee (3) suggested that the streaks resulted from a toxin produced by the fungus and showed that culture filtrate from *H. sacchari* was toxic to excised sugarcane leaves. He attributed the toxicity to nitrite accumulations in the medium.

Our studies were initiated to determine more fully the cause for toxicity of culture filtrate, to check for host specificity of the toxin, and to determine the feasibility of using toxin in screening clones for resistance to eye spot disease. Brief reports of some of this work have been published (11, 12).

MATERIALS AND METHODS.—*Helminthosporium sacchari* was maintained on cane leaf agar (14). For toxin production, the fungus was grown for 20-24 days in still culture in 1-liter Roux bottles containing 150 ml of modified liquid Fries' basal medium (5) supplemented with 0.1% yeast extract. Richards medium was used in an experiment attempting to verify the findings of Lee (3). The modified colorimetric method of Gress-Ilosvoy was used to determine nitrite concentration in the culture medium (1).

Purification procedures.—Methods similar to those used by Pringle & Scheffer (10) were used to partially purify the toxin. The fungus was removed from the medium by straining through cheesecloth and filtering through No. 1 and 50 Whatman filter papers. The filtrate was concentrated to one-tenth the original volume under vacuum at 42 C. An equal volume of methanol was added, and after 24-48 hr at 4 C the precipitate was removed by filtration and discarded. Methanol was removed from the supernatant liquid by evaporation, and the remaining solution was partitioned against three volumes of chloroform, each volume equal to the toxin solution volume. The aqueous phase was concentrated to one-fiftieth of the original volume. Further purification was accomplished by extracting 1 ml of the concentrated aqueous phase with 3 successive 10-ml volumes of water-saturated butanol. After evaporation of the butanol, the toxic material was suspended in 1 ml water, and added to an 18.0×1.3 cm Sephadex G-15 column. Toxin was eluted from the column with water. Toxin recovered from peaks 1 and 2 were used in the data reported herein (Fig. 1-A).

Assays.—Two assay procedures were used to determine toxin activity. In the first, 7-inch excised leaves were used. A 1-μliter drop of toxin solution was placed on a needle-puncture spot near the base of the leaf. Injected leaves were incubated in a moist chamber at room temperature. The length of the resulting runner from each of several toxin dilutions was an indication of toxin activity and concentrations (Fig. 2-A). Measurements were made 24-48 hr after inoculation. In the second, 0.2 ml of toxin solution was injected into the leaf spindle immediately above the apical meristem of intact plants (Fig. 2-B). Degrees of clonal resistance could be determined by using several dilutions of toxin and rating the intensity of the symptoms from each dilution. Ratings were determined 24-30 hr after injection. A modification of the second procedure consisted of injecting 0.2 ml of toxin solution into a 5-mm hole in the stalk made with a No. 2 cork borer.

Fungus inoculations were made according to the method of Wismer & Koike (14). The upper four to six leaves of a stalk were sprayed with a spore suspension and covered with a plastic bag for 7 days. Ratings of the fungus reactions were determined 10-20 days after inoculations.

RESULTS.—*Activity of partially isolated toxin.*—A filtrate from 24-day-old cultures did not differentiate between resistant and susceptible clones of sugarcane. Staling products were probably responsible for the nonspecific reaction. When the filtrate was diluted 10 times, a resistant clone, H 50-7209, was unaffected

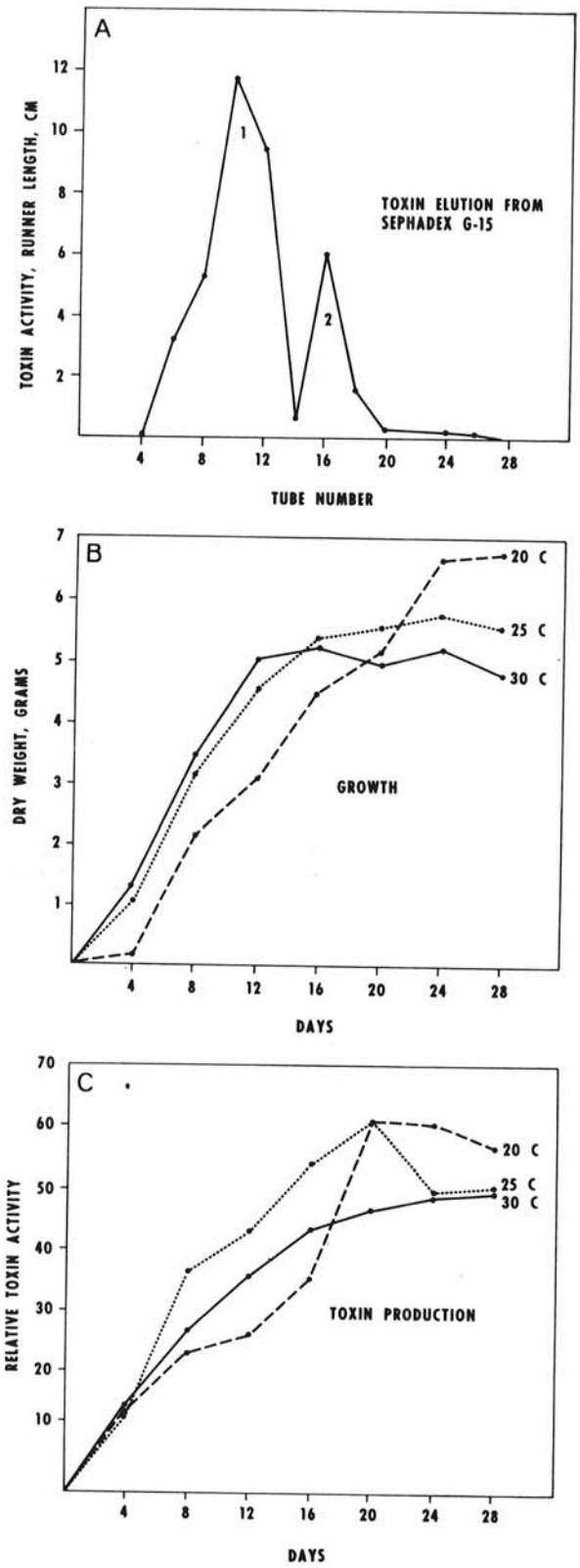

Fig. 1. A) Elution of toxin from *Helminthosporium sacchari* from a Sephadex G-15 column. One ml of toxin solution, equivalent to 50 ml of crude filtrate, was added to the column and collected in 1-ml fractions. Toxin activity was determined by placing drops on needle punctures near the base of a leaf. **B)** Growth of *Helminthosporium sacchari.* Each point represents the total dry weight from three 250-ml Erlenmeyer flasks, each containing 50 ml of modified Fries' medium. **C)** Toxin production of *Helminthosporium sacchari.* Toxin activity was determined by placing drops on needle punctures near the base of the leaf. Activity was based on runner length and symptom intensity with each criteria rated between 1 and 9, 1 representing no activity. Two leaves having four needle punctures were used for each of four tenfold dilutions. Each point was determined by totaling the average runner length and symptom intensity from the four dilutions.

when drops were placed on needle punctures near the base of the leaf. Runners less than 3.0 cm long were produced on a susceptible clone, H-109. After the toxin solution was partially purified with solvents and gel filtration, runners at least 3.0 cm long resulted from approximately 0.5 μg/μliters of toxin solution on the susceptible clone. High concentrations of toxin produced runners that extended the entire length of the 7-inch leaves. These reddish-brown symptoms were easily detected within 8-12 hr. The color of the runner is similar to symptoms produced by the fungus (Fig. 3). Clone H 50-7209 was unaffected by the fractionated toxin.

Nitrite could not be detected in the crude filtrate or fractionated solution when Richards, the medium used by Lee (3), or Fries' medium was used. Nitrite in concentrations up to 50 μg/μl liter caused no toxic reactions on leaves of H-109 or H 50-7209 when drops were placed on a needle puncture near the base of the leaf. A concentration of 10,000 μg/ml caused no visible symptoms on either clone when the toxin was injected into the stalk. It was concluded that a toxin other than nitrite was responsible for symptoms on H-109.

Toxin production and stability.—The addition of 0.1% yeast extract to Fries' medium increased toxin production by twofold. The toxin produced from either medium was host-specific, affecting only susceptible sugarcane clones.

Toxin production and fungus growth were affected by both temperature and time (Fig. 1-B,C). The fungus grew faster at 30 C than at 20 and 25 C. Toxin production at 30 C was less than at 20 and 25 C. Maximum production was obtained between 16 and 20 days at 20 and 25 C.

During the 20-day incubation the pH of the medium changed from 5.5 to approximately 3.8. At the lower pH, the partially purified toxin (methanol- and chloroform-treated) is stable. A preparation tested periodically on several clones did not lose activity over 352 days (Table 1).

Similarly prepared toxin is also stable to heat. Toxin was heated in a sealed glass container for 30 min. At temperatures from 50 to 144 C the toxin was unaffected when its activity was determined by placing drops on needle punctures near the leaf base on H-109. Activity was greatly reduced at temperatures higher than 144 C. Clone H 50-7209 was unaffected with toxin heated at any of the temperatures.

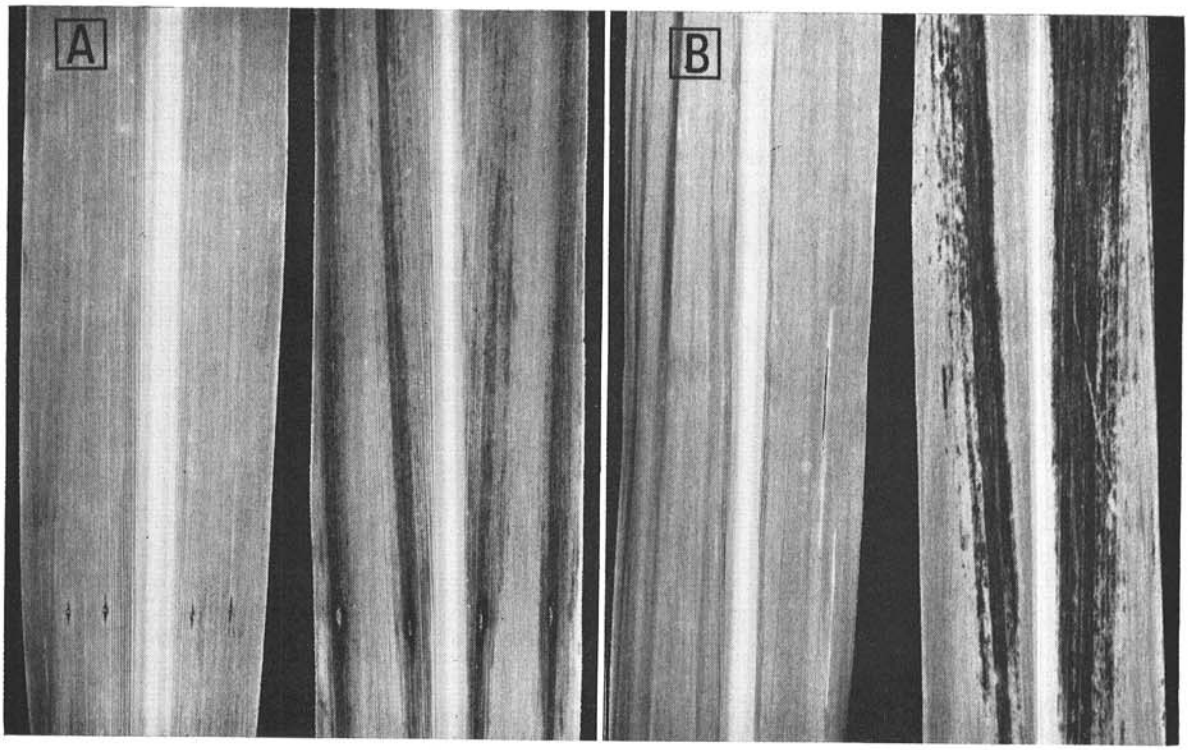

Fig. 2. Assays used to determine toxin activity. **A)** A 1-μliter drop of toxin solution was placed over a needle-puncture hole. The leaf on the left was from a resistant clone and the leaf on the right from a susceptible clone. **B)** A toxin solution was injected into either the spindle or stalk. The leaf on the left was from a resistant clone, the leaf on the right from a susceptible clone.

Host specificity.—Fifteen clones of sugarcane were tested to determine their reaction to the toxin and *H. sacchari* (Table 2). These clones were known to differ in their resistance to *H. sacchari.* Three concentrations of fractionated toxin (1:10, 100, 1000) were used with both assay procedures. An international rating scale (2) of 1 to 9, with 1 signifying a resistant clone, was used to rate the reaction to the toxin and fungus. In laboratory assays, runner lengths from the three toxin concentrations were totaled and divided by a number which made the divided total for H-109 equal to 8. The reaction of the clones to toxin injected into the stalk was based on the number of leaves affected and symptom intensity. Ratings for the three toxin concentrations were totaled and divided by a number which made H-109 equal to 8. Results indicate that clones susceptible, intermediate, or resistant to the fungus react in a similar way to the toxin.

TABLE 1. Effect of time on activity of toxin from *Helminthosporium sacchari* on four clones of sugarcane using the spindle injection technique[a]

Toxin age, days	Clonal reactions[b]			
	H-109	H 54-775	H 58-8029	H 50-7209
15	8	5	8	1
45	8	4	7	1
130	8	5	7	1
352	8	6	7	1

[a] Toxin solution was stored at 4 C and had a 4.2 pH.
[b] Based on a scale of 1 to 9 basis, with 1 indicating a resistant clone, 9 a highly susceptible clone.

TABLE 2. Reaction of 15 clones of *Saccharum* sp. to *Helminthosporium sacchari* inoculations and toxin produced by this pathogen

Clone	Clonal reactions[a]		
	Leaf spot[b]	Spindle injections[c]	Fungus inoculations
H-109	8	8	8
Lahaina	8	8	8
H 37-1933	3	5	4
H 49-5	1	1	2
H 49-823	5	6	7
H 50-7209	1	1	1
H 52-4610	1	1	3
H 54-775	5	5	5
H 57-4114	2	1	2
H 57-1627	[d]	3	3
H 57-5174	1	1	1
H 58-8029	6	6	7
H 61-2797	5	6	8
H 61-2862	2	3	2
H 62-2145	2	3	1

[a] Based on a scale from 1 to 9, with 1 indicating a resistant clone and 9 a highly susceptible clone.
[b] A 1-μliter drop of toxin placed on a needle-puncture wound of an excised cane leaf.
[c] 0.2 ml of toxin solution.
[d] H 57-1627 was not rated due to atypical runner symptoms.

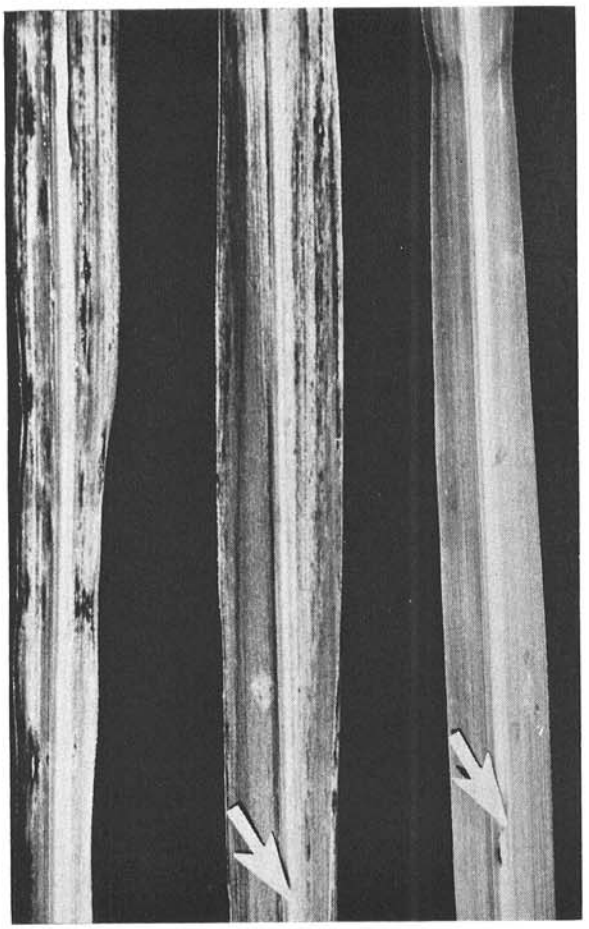

Fig. 3. Comparative symptoms of leaf reactions to *Helminthosporium sacchari* and toxin. Toxin was injected into the midrib at the arrow point with a syringe. The leaf on the left and center were from the susceptible clone H-109, inoculated with a spore suspension and toxin, respectively. The leaf on the right was from clone H 50-7209 and was resistant to the toxin and fungus.

Toxin and *H. sacchari* were both tested on the following plants: sweet and field corn, pea, bean, tomato, sudan grass, sorghum, alfalfa, and barley. All excised plants were unaffected by approximately 50 μg/μliter of fractionated toxin using drops on a puncture near the leaf base. When leaves or stems were placed in toxin solution, plants were unaffected with 10 μg of fractionated toxin/μliter. The fungus caused no symptoms on any of these species except on sorghum, where small flecks were formed. These results clearly indicate that the toxin produced by *H. sacchari* is host-specific, affecting only plants to which the fungus is pathogenic.

Screening sugarcane clones for resistance to eye spot disease with toxin.—Preliminary laboratory and field tests with 15 commercial clones indicated that toxin could be used to screen for resistance to eye spot disease. Additional tests in the field were made to verify these results. Toxin was injected into the stalk rather than the leaf spindle, since this allowed a consistent quantity of toxin to be easily introduced into the plant. All clones were injected with the same preparation of methanol- and chloroform-treated toxin. The fungus used to inoculate the clones was the same isolate that produced the toxin.

A total of 182 clones, 47 developed in Hawaii and 135 imported, was tested. There was a highly significant correlation ($r = .88$, $P < .01\%$) between the reaction of fungal inoculations and reaction to the toxin. We concluded that the reactions of a sugarcane clone to the toxin is indicative of its reactions to the fungus.

A variation of three numbers on the rating scale was noted in only 4 clones; 29 clones varied by two numbers. Of these, 23 were more susceptible to toxin than to the fungus while 10 were more susceptible to the fungus. These clones were retested and similar results were obtained. Reasons for these variations have yet to be determined.

DISCUSSION.—Pringle & Scheffer (9) defined a host-specific toxin as a metabolic product of a pathogen which is toxic only to the host of that pathogen. The toxin produced by *H. sacchari* has several characteristics in common with the other host-specific toxins: (i) the host range of the toxin is similar to the fungus; (ii) the symptoms produced by the toxin and fungus are similar; and (iii) the toxic compound has a low mol wt as indicated by the elution pattern on a Sephadex G-15 column (9). It is similar to *H. carbonum* toxin in that it is stable to high temperatures (10); this, plus its ability to remain active over extended periods of time, should aid in its isolation and further characterization.

Toxin production was greater at 20 C than at 30 C, and thus it might be expected that *H. sacchari* would be more pathogenic at lower temperatures. Liu (4) showed that *H. sacchari* is more pathogenic at 20 C than at 30 C even though growth of the fungus was more rapid at 30 C. In Hawaii, C. A. Wismer, of the Hawaiian Sugar Planters' Association (*personal communication*) also noted that cool wet climates contribute to incidence of eye spot disease.

The elution curve of the toxic filtrate on Sephadex G-15 indicates that two toxins are present (Fig. 1-A). It would appear that both are host-specific, since both fractions were included in the toxin preparations used in this study.

Screening for resistance to eye spot disease is possible using this host-specific toxin (12). The use of other host-specific toxins to screen for disease resistance has been suggested (8). Wheeler & Luke (13) reported a technique for screening large numbers of oat seedlings for resistance to victoria blight using the host-specific toxin produced by *H. victoriae*. R. P. Scheffer, Michigan State University (*personal communication*), indicated that toxin could be used to screen for resistance to the leaf spot disease of corn caused by *H. carbonum*. Several factors are important in obtaining consistent results from toxin injections for screening clones resistant to eye spot disease: (i) clones of the same age should be used since the rating system is based on a comparative scale; (ii) stalks used should be of sufficient size to allow 0.2 ml of toxin solution to be injected; and (iii) stalks of different

stools should be used for testing different dilutions, since in a few cases toxin was translocated downward and into another stalk within the same stool.

Using the toxin to screen for eye spot disease resistance has certain advantages over using fungal inoculations. Reactions to fungal inoculations are more sensitive to environmental influences than are reactions to toxin injections. Results of toxin reactions can be obtained in 24 hr. Possible changes in virulence of the pathogen in culture are eliminated, as large quantities of toxin can be made and stored for use over extended periods of time. Countries not yet having *H. sacchari* present can determine the reaction of their clones to eye spot disease without introducing the pathogen. Several countries are currently testing their clones against "foreign diseases" by sending the clones to areas where the diseases are already present, but a 1- to 2-year quarantine period is required before they can be tested. Testing with toxin would eliminate these time delays and many more clones could be tested.

Clones differ in their reaction to *H. sacchari* in different areas of the world (7). Environmental and strain differences could account for this. Strain differences could be studied by exchange of toxin preparation, thus eliminating the risk of introducing new strains of the pathogen.

Because of its host specificity, its stability, and the similarity of its reaction to disease symptoms, *H. sacchari* toxin appears to be an ideal model system for further study of host-parasite interactions.

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