Attenuation of pathogenicity of Helminthosporium sacchari
by Frederick David Pinkerton

A thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Plant Pathology
Montana State University
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Abstract:
Helminthosporium sacchari (Van Breda de Haan) Butler, the casual agent of the eye-spot disease of sugarcane, produces a host-specific phytotoxin that is required for pathogenesis. If the fungus is maintained on a synthetic medium it loses the ability to produce toxin in culture, i.e., it becomes attenuated. When the attenuated strain is transferred back to sugarcane extract agar, it regains the ability to produce toxin in culture. This indicates that the fungus requires a compound(s) found in susceptible sugarcane for toxin production. The present study describes the systematic acquisition of attenuated strains of H. sacchari (those that do not produce toxin in culture), and the isolation and characterization of one of several "activator" compounds from sugarcane that will activate toxin biosynthesis in cultures of attenuated H. sacchari.

Attenuated strains of H. sacchari were obtained by successively subculturing a pathogenic strain of H. sacchari on a synthetic medium until toxin production in culture ceased. In two experiments, the number of transfers required to achieve attenuation was six.

Activator was purified from rinse water obtained by washing leaves of susceptible sugarcane with distilled water. A combination of cation exchange chromatography, paper chromatography, and high voltage paper electrophoresis provided a white powder whose melting point showed initial decomposition at 91° and final decomposition at 214° . On the basis of infra red, ultra violet, and mass spectral data, the chemical name 2-amino-1,3-propanediol was assigned to activator. The compound was given the trivial name serinol. A synthetic compound was prepared which had nearly identical chemical, physical, spectral, and biological properties to those of the natural activator.

In assay cultures, activator had a concentration of optimum activity of 10-6 M. Above and below this concentration activity fell to zero. Activator, at a concentration of 10 -6M, was added to cultures derived from single spore and hyphal tip isolates of attenuated H. sacchari. Activation (toxin production) occurred in 53% of the single spore cultures and in 78% of the hyphal tip cultures. Activation did not occur in any of the cultures that did not contain activator.

Activator, which probably originates in the host, may play a role in the natural host parasite system. Attenuated strains of H. sacchari resume toxin production and assume a pathogenic mode on susceptible sugarcane if they are provided a moist environment for a sufficient period of time. Sugarcane resistant to the fungus does not contain activator on or within the leaves. Hence, resistant sugarcane is not capable of activating attenuated strains of H. sacchari.

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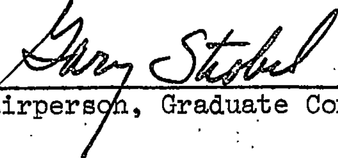
Approved:



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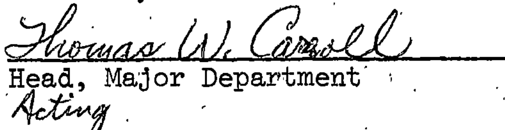

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TABLE OF CONTENTS

	Page
VITA	ii
ACKNOWLEDGEMENTS	iii
LIST OF TABLES	vi
LIST OF FIGURES	viii
ABSTRACT	x
Chapter	
1. INTRODUCTION	1
2. MATERIALS AND METHODS	9
BIOLOGICAL MATERIALS	9
Sugarcane	9
Cultures	9
Helminthosporoside Production	10
Bioassay for Helminthosporoside	11
Hyphal Tip and Single Spore Isolates from Attenuated Cultures	11
Hyphal tip isolates	11
Single spore isolates	12
Isolation of Sugarcane Leaf Microflora	12
In vivo Inoculation with Attenuated _H. sacchari_	14
PURIFICATION AND CHARACTERIZATION OF ACTIVATOR	14
Chromatography	14
Cation Exchange Chromatography	15
High Voltage Paper Electrophoresis	15
Purification of Activator	16
Leaf wash method	16
Leaf extraction method	17
Instrumental Analysis	17
Synthesis of 2-amino-1,3-propanediol	18
3. RESULTS	19
Acquisition of Attenuated Variants of _H. sacchari_	19

TABLE OF CONTENTS (continued)

Chapter Page

Purification of Activator. 22
Properties of Activator. 26

BIOLOGICAL PROPERTIES OF ACTIVATOR 32
Optimal Activity of Activator. 32
Hyphal Tip and Single Spore Isolates:
Interaction with Activator 32
Microflora as a Source of Activator. 36
In Vivo Inoculation with Attenuated H. sacchari. . . 38
Host Range of Activator. 41
Synthetic Activator. 43

4. DISCUSSION 51

5. SUMMARY. 68

LITERATURE CITED 70

LIST OF TABLES

Table		Page
1.	Number of transfers required for attenuation of pathogenic *H. sacchari* in culture. The (+) and (-) signs indicate the results of bioassays conducted on the M1D cultures at each step in the flow diagram in Fig. 1.	21
2.	Helminthosporoside (nmols) produced in assay culture at each step in purification of activator. . . .	24
3.	R_f and $R_{a.a.}$ values of activator. Solvent a was used for descending paper chromatography and solvents b-g were employed for thin layer chromatography.	25
4.	Absorption bands and assigned functional groups from the infra red spectrum of natural activator, Fig. 3.	28
5.	Toxin production in cultures of attenuated *H. sacchari* as a function of time and natural activator concentration.	33
6.	Activation of cultures derived from single spore and hyphal tip isolates from an attenuated strain of *H. sacchari*.	35
7.	Activation of cultures of attenuated *H. sacchari* by washate and extract from alcohol treated leaves. . . .	37
8.	Activation of cultures of attenuated *H. sacchari* by culture filtrates of organisms isolated from sugarcane leaves.	39
9.	The ability of extracts of various canes to activate toxin production in cultures of attenuated *H. sacchari*.	42
10.	Activation of cultures of attenuated *H. sacchari* by leaf wash from resistant sugarcane.	44
11.	R_f values of synthetic and natural activator.	46

LIST OF TABLES (continued)

Table Page

12. Bioassay of synthesized activator 49

13. Toxin production in cultures of attenuated *H. sacchari* as a function of synthetic activator concentration 50

LIST OF FIGURES

Figure		Page
1.	Flow chart summarizing the procedure for detecting attenuation in culture. A pathogenic strain was successively subcultured on synthetic M1D medium until toxin could not be detected by bioassay. For procedural details see text.	20
2.	Flow diagram illustrating the procedure used to purify activator. Location of biological activity was determined indirectly using the helminthosporoside (toxin) leaf bioassay.	23
3.	Infra red spectrum of natural activator. Analysis was conducted on a Beckman Microspec spectrophotometer using a KBr micropellet.	27
4.	Low resolution mass spectrum of natural activator. The spectrum was obtained on a LKB Model 9000 spectrometer with 20 eV on the filament and the probe heated to 80°	30
5.	Proposed structures of some of the major peaks in the mass spectrum of natural activator, Fig. 4	31
6.	In vivo inoculation of susceptible leaves with pathogenic and attenuated strains of H. sacchari. In photo A, the leaf on the left was inoculated with a pathogenic strain and the leaf on the right with an attenuated strain. The leaves were wrapped in plastic sacs for 24 hr. The leaf in photo B was inoculated with an attenuated strain and wrapped in a plastic sac for 48 hr	40
7.	Infra red spectrum of snythetic activator. Analysis was conducted on a Beckman Microspec spectrophotometer using a KBr pellet	47
8.	Low resolution mass spectrum of synthetic activator. The spectrum was obtained on a LKB 9000 spectrometer with 20 eV on the filament and the probe heated to 80°	48

LIST OF FIGURES (continued)

Figure Page

9. Elution profile of the cation fraction of sugarcane extract from a Bio-Gel P-2 column, 1.5 x 48 cm. One ml fractions were collected, $V_o = 32$ 53

10. The proposed structure of activator, 2-amino-1,3-propanediol 55

ABSTRACT

Helminthosporium sacchari (Van Breda de Haan) Butler, the casual agent of the eye-spot disease of sugarcane, produces a host-specific phytotoxin that is required for pathogenesis. If the fungus is maintained on a synthetic medium it loses the ability to produce toxin in culture, i.e., it becomes attenuated. When the attenuated strain is transferred back to sugarcane extract agar, it regains the ability to produce toxin in culture. This indicates that the fungus requires a compound(s) found in susceptible sugarcane for toxin production. The present study describes the systematic acquisition of attenuated strains of H. sacchari (those that do not produce toxin in culture), and the isolation and characterization of one of several "activator" compounds from sugarcane that will activate toxin biosynthesis in cultures of attenuated H. sacchari.

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Activator was purified from rinse water obtained by washing leaves of susceptible sugarcane with distilled water. A combination of cation exchange chromatography, paper chromatography, and high voltage paper electrophoresis provided a white powder whose melting point showed initial decomposition at 91° and final decomposition at 214°. On the basis of infra red, ultra violet, and mass spectral data, the chemical name 2-amino-1,3-propanediol was assigned to activator. The compound was given the trivial name serinol. A synthetic compound was prepared which had nearly identical chemical, physical, spectral, and biological properties to those of the natural activator.

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INTRODUCTION

Inherent in any plant host-parasite system is the ability of the parasite to invade and perpetuate itself within the host population. This ability to cause disease is the resultant of a three way interaction between the host, the parasite and the environment. A change in one component of this interaction may alter drastically the pathogen's success.

The capability of plant parasites to change in response to alterations in the host or the environment is well known. The terms pathogenicity, virulence, and aggressiveness have been used in different ways to describe various attributes of the ability to cause disease (22, 44, 45). Regardless of usage, implicit in each term is the concept of variability.

Variation within a pathogen population may produce highly efficient strains that are extremely successful parasites. Conversely, strains may arise in which the ability to persist parasitically is greatly reduced or eliminated. This loss of parasitic capabilities has been labeled attenuation (4). Although the term originally referred to alterations in viruses during host passages (4), attenuation here describes a reduction or loss of the capacity to carry on a parasitic mode of existence.

Numerous examples of attenuation among bacteria and fungi exist in the literature. The phenomenon is most commonly observed in the laboratory but attenuation also occurs in nature. A survey of these

examples reveals that two types of attenuation exist.

The first type represents an allelopathic (47) interaction between two strains of a parasite engaged in intraspecific competition. For example, Van Alfen, et al. (43) demonstrated that a cytoplasmic factor transferred by hyphal anastomoses from a hypovirulent strain to a virulent strain of *Endothia parasitica* attenuated the virulent strain to a state of hypovirulence. Transfer of the factor occurs both in host tissue and in culture. The nature and mode of action of the cytoplasmic factor is unknown but the ability of the attenuated strain to invade its host, the American Elm, is reduced.

A similar example comes from the study of crown gall, the disease incited by *Agrobacterium tumefaciens*. Kerr and Htay (16) established that a non-pathogenic variant, *Agrobacterium radiobacter* var. *radiobacter* attenuated a pathogenic variant, *Agrobacterium radiobacter* var. *tumefaciens*, by releasing the proteinaceous antibiotic bacteriocin 84. The colonies of *A. tumefaciens* that survived were resistant to bacteriocin 84 but were no longer pathogenic. Roberts and Kerr (24) postulated that a specific molecular configuration on the bacterial surface is necessary for pathogenesis and that this configuration is a receptor for the antibiotic. Knowledge of this attenuation mechanism has led to the biological control of crown gall of peach in Australia (15).

The second type of attenuation originates from mechanisms intrinsic to the reproductive system utilized by the organisms

involved. In sexually reproducing plant pathogens, attenuation may arise by mutation or by various recombination mechanisms. Pathogens reproducing asexually become attenuated via several mechanisms: mutation, heterokaryosis, parasexuality, or physiological adaptation (4, 46).

Regardless of the mechanism(s) responsible, attenuated variants generally fall into two categories: 1) Those which are attenuated because of specific biochemical lesions causing nutritional abnormalities, and 2) those which are attenuated for no discernible reason.

Variants in the first category require specific substances for expression of pathogenicity. A tryptophan requiring mutant of _Erwinia aroideae_ that was avirulent on potato has been described by Garber and Hackett (9). Garber, et al. (10) also described avirulent mutants of _Agrobacterium tumefaciens_ that varied in their nutritional requirements. One mutant, requiring only tryptophan, was totally avirulent when challenged with its _Bryophyllum_ host. Mutants requiring both adenine and methionine exhibited variable virulence.

During their monumental study of the genetics of _Venturia inaequalis_, Keitt and his colleagues induced a series of biochemical mutants that varied in pathogenicity. Those mutants requiring amino acids, the nitrogen bases, choline, or riboflavin were non-pathogenic (2). If the non-pathogenic mutants were incubated with the required substances on apple leaves, all the variants except those requiring

the purine bases were capable of inciting disease. Apparently these mutants could not obtain sufficient substrates from their hosts to incite disease under natural conditions (17).

The foregoing examples illustrate a form of attenuation in which well defined biochemical lesions are responsible for the loss of pathogenicity. No such clearly defined explanations are available for the second category of attenuated variants. This category represents an incredibly diverse group of plant pathogenic bacteria and fungi which, for no apparent reason, attenuates in the field or, more commonly, in laboratory culture. No attempt will be made to review the voluminous literature available. Rather, several members of the genus *Helminthosporium* will be discussed; *Helminthosporium sacchari* in detail.

The genus *Helminthosporium* includes the conidial stages of pathogens responsible for serious diseases of rice, corn, grasses, and cereals (6). Several members of the genus, including *H. victoriae*, *H. carbonum*, *H. maydis* and *H. sacchari*, produce host specific toxins. These compounds are fungal metabolites toxic only to the host susceptible to the pathogens producing them. Compounds of this type induce virtually all disease symptoms and are critical for pathogenesis (37).

Pathogenicity in these fungi is dependent upon their host specific toxins. Hence, loss of toxin production and attenuation are synonymous. Attenuation in the toxin producing helminthosporia occurs with maddening frequency. Karr, et al. (14) were forced to perpetuate

H. maydis on susceptible corn leaves to maintain pathogenicity. Loss of pathogenicity in cultures of H. carbonum and H. victoriae prompted Scheffer, et al. (25) to comment:

> "The survival of the gene conferring toxin production in the absence of known toxin-sensitive susceptible plants is a problem deserving special attention. A satisfactory understanding of this problem could contribute to our knowledge of epidemiology, plant disease ecology, and the biochemistry of pathogenicity."

Study of another toxin producing member of the genus Helminthosporium has provided some insight into this problem

Helminthosporium sacchari (Van Breda de Haan) Butler is the casual agent of the eyespot disease of sugarcane. This disease, found in most areas where sugarcane is grown (20), is characterized by the formation of eye shaped lesions, followed by the development of reddish-brown "runners" which extend from the lesion toward the leaf tip.

The fungus can be isolated from the eye shaped lesions but not from the runner areas of dead tissue (20). This observation prompted the hypothesis that a toxin secreted by the fungus in the primary lesion was responsible for runner formation. Lee (18) demonstrated that the filtrate of fungal cultures contained a toxic substance which destroyed chlorophyll and reduced the iron in leaves. Steiner and Byther (31) partially purified a toxin from culture filtrates that elicited disease symptoms and exhibited the same host range as the fungus. Steiner and Strobel (32) subsequently purified the toxin and

characterized it as 2-hydroxycyclopropyl-α-D-galactopyranoside. The trivial name helminthosporoside was assigned on the basis of its source and structure.

Helminthosporoside is directly involved in the pathogenesis of eyespot disease. Steiner and Byther (31) found that the reactions of 182 clones of cane to the toxin and to the fungus were significantly correlated (r = 0.88). Strobel (35) demonstrated that the amount of toxin bound to a component of membrane preparations of 28 clones was directly related to the reaction of those clones to toxin. He subsequently showed that similar plasma membrane proteins were present in both susceptible and resistant clones (35). The protein of susceptible clones bound the toxin whereas the protein of resistant clones did not. Amino acid analysis revealed that the immunologically identical proteins differed by one amino acid residue each of lysine, glutamate, serine and glycine (36). He postulated that resistance to eyespot disease is conferred by an altered toxin binding protein.

The physiological function of the binding protein is thought to be α-galactoside transport (38). The α-galactosides raffinose, melibiose, and methyl-α-galactopyranoside are bound by the protein (resistant clones exhibit no binding) but disease symptoms do not appear. Binding alone cannot initiate pathogenesis.

Strobel (38) demonstrated that binding of toxin activated a membrane bound ATPase and postulated that binding of toxin altered the

binding protein's structure to such an extent that the ATPase was activated by a resulting perturbation in the membrane. The increased ATPase activity upset the cell's osmotic balance which resulted in lysis and death. This hypothesis accounts for the observed water clearing and the appearance of water droplets on the leaf surface. These droplets have a low water potential due to the presence of solutes (amino acids and sugars) which is expected if the membranes have lost control of influx and efflux of molecules.

The above evidence demonstrates that helminthosporoside is a necessary disease determinant. It is required for fungal colonization and is responsible for disease symptoms. Pathogenicity of *H. sacchari* is thus defined in terms of toxin biosynthesis. Those variants that cannot biosynthesize the toxin are non-pathogenic--they are attenuated.

H. sacchari, when successively subcultured on synthetic medium, becomes attenuated, i.e., it ceases to produce helminthosporoside in culture. If the attenuated strain is transferred back to cane infusion agar, toxin biosynthesis is restored in culture. This suggests that something in the cane is required for toxin biosynthesis—a substance that "activates" helminthosporoside biosynthesis in an attenuated strain of *H. sacchari*.

This report describes the systematic acquisition of attenuated variants of *H. sacchari* and the isolation, purification, and chemical

characterization of one of several "activators" from sugarcane that can restore toxin biosynthesis in these variants. Some discussion of the importance of this phenomenon to the plant host-parasite system is also presented.

MATERIALS AND METHODS

BIOLOGICAL MATERIALS

Sugarcane

Susceptible (51 NG 97) and resistant (H 50-7209) clones of sugarcane were acquired from Dr. R. Coleman, United States Department of Agriculture, Beltsville, MD. Stalks were planted in large plastic buckets and grown at 22 $\pm$ 5° under greenhouse conditions. Mature leaves used in this study were removed from the upper portions of the stalks.

Cultures

Pathogenic strains of H. sacchari were provided by Dr. Gary Steiner, Hawaiian Sugar Planters Association, Honolulu, Hawaii. The strains were maintained on slants of cane leaf agar prepared by extracting leaf sections (1 g tissue/5 ml H_2O) for 15 min at 78° in an isothermal autoclave, filtering the extract through eight layers of cheesecloth, and reducing the volume to 0.25 of the original. Agar was added to a final concentration of 2 g/100 ml.

A pathogenic strain of H. sacchari was successively subcultured on M1D medium until attenuated (see results). The attenuated strain was sustained on agar slants of the same medium. The medium, a modification of the M1D medium described by Filner (8), consisted of $Ca(NO_3)_2$, 1.2 mM; KNO_3, 0.79 mM; KCl, 0.87 mM; $MgSO_4$, 3.0 mM;

NaH_2PO_4, 0.14 mM; sucrose, 87.6 mM; ammonium tartrate, 27.1 mM; $FeCl_3$, 7.4 µM; $MnSO_4$, 30 µM; $ZnSO_4$, 8.7 µM; H_3BO_3, 22 µM; and KI, 4.5 µM. The pH was adjusted to 5.5 with 0.1 N HCl. Slants of both strains were stored at room temperature.

Helminthosporoside Production

Approximately 0.1 g of mycelium from an attenuated strain of *H. sacchari* and 20 ml of deionized, distilled H_2O were placed in the cup of a Sorvall Omnimixer and ground at top speed for 15 sec. A 5 ml portion of the suspension was transferred by Pasteur pipet to a 50 ml Erlenmeyer flask containing 10 ml of modified M1D medium. All operations were carried out under sterile conditions.

The cultures were grown for 7 to 10 days in a Percival incubator at 28 $\pm$ 1° under 500 ft candles of continuous cool, white fluorescent light.

Cultures were assayed for the presence of helminthosporoside by modification of the procedures developed by Steiner and Strobel (32). The fungal mat was removed from the medium by filtration through four layers of cheesecloth. Three volumes of acetone at -15° were added to the filtrate while stirring. Centrifugation at 20,000 x g for 10 min removed the precipitate. Acetone was removed by evaporation under vacuum at 45° and the remaining aqueous solution extracted three times with equal volumes of cold water saturated n-butanol. The n-butanol

phases were combined and the n-butanol evaporated to dryness under vacuum at 60°. The remaining residue was dissolved in deionized, distilled H_2O and bioassayed for the presence of helminthosporoside.

Bioassay for Helminthosporoside

Bioassays were performed on leaves from the susceptible clone 51 NG 97, utilizing a variation of methods described by Steiner and Byther (31). Leaf sections 18 cm long were punctured on either side of the midrib near the base and 1 µl of test solution placed on the wounds. The leaves were placed in a plastic crisper box and held at room temperature for 22 hr at which time symptom development was evaluated. Symptoms were quantitated by measuring runner length from the point of inoculation to the tip of the runner as it had developed up the leaf. Runner formation below the point of inoculation was not measured.

Hyphal Tip and Single Spore Isolates from Attenuated Cultures

Hyphal tip isolates. Discs of attenuated mycelium were cut with a sterile 8 mm cork borer and transferred to 150 x 15 mm petri plates containing M1D agar. The discs were incubated for 3 days at room temperature.

A sterile glass needle was used to transfer hyphal tips from the fine mycelial growth surrounding the discs to 50 x 12 mm petri plates

of M1D agar. The plates were incubated for 7 days at 28 $\pm$ 1° under 500 ft candles of continuous cool, white fluorescent light.

The mycelial contents of each plate were transferred to 50 ml Erlenmeyer flasks containing 10 ml of M1D medium and incubated as described preceeding bioassay for helminthosporoside.

Single spore isolates. A sporulating culture of attenuated *H. sacchari* was washed gently with a 0.1% solution of Tween-20. This procedure detached conidia without disturbing the mycelial mat. The conidial suspension was added to several 150 x 15 mm petri plates which were swirled to assure uniform dispersal of conidia over the M1D agar. Following a 24 hr germination period, single, viable spores were transferred to M1D agar in 50 x 12 mm petri plates. The plates and their contents were then treated in the same manner as the hyphal tip cultures.

Isolation of Sugarcane Leaf Microflora

Fungal and bacterial components of sugarcane leaf microflora were isolated and cultured on Eckert's modified medium (41) and Medium 523 of Kado, et al. (12), respectively.

Eckert's medium contained glucose, 99.9 mM; K_2HPO_4, 8 mM; KH_2PO_4, 12.4 mM; $MgSO_4$, 2 mM; 0.3% Yeast Extract; and Difco Peptone, 0.5%.

Medium 523 consisted of sucrose, 29.2 mM; K_2HPO_4(An.), 11.4 mM;

$MgSO_4 \cdot 7\ H_2O$, 1.2 mM; Casein acid hydrolysate, 0.8%, and Yeast Extract, 0.4%.

Leaf segments 3 cm long were either surface sterilized in 5% sodium hypochlorite for 3 min or used without sterilization. The segments were imprinted in 100 x 17 mm petri dishes containing the aforementioned media solidified with 2% agar and incubated at $30 \pm 1^{\circ}$ under 500 candles of continuous cool, white fluorescent light.

Individual colonies of each organism present were isolated and subcultured on the appropriate medium until pure. Classification of the organisms was not attempted.

Bacteria were inoculated into 1 liter Erlenmeyer flasks containing 100 ml of Medium 523 and the cultures grown for three days in the dark at $30 \pm 1^{\circ}$ in a rotary shaker-incubator at a rotation speed of 155 rpm.

Centrifugation at 20,000 x g for 15 min removed bacteria from the medium. The cell free medium was adjusted to pH 5.5 with 1 N HCl, decanted into 500 ml Roux flasks, and autoclaved for 15 min at 132°. The flasks were inoculated with attenuated _H. sacchari_ as described above. The cultures were grown at room temperature for 21 days.

Fungi were inoculated into 500 ml Roux flasks containing 150 ml of Eckert's modified medium and grown at room temperature for 18 days.

The mature fungal mats were removed from the medium by straining through several layers of cheesecloth. The medium was filtered through Whatman No. 1 filter paper and subsequently treated in the same manner

as the bacterial medium.

Following the 21 day growth period, the H. sacchari cultures were harvested and the filtrate purified and bioassayed for helminthosporoside.

In Vivo Inoculation with Attenuated H. sacchari

Stalks of susceptible cane supporting 5 to 7 leaves were placed in 1 ℓ Erlenmeyer flasks containing 500 ml of Hoglands' solution.

Suspensions of attenuated and pathogenic strains of H. sacchari were prepared by scraping approximately 0.2 g of mycelium from culture plates with a spatula and mixing thoroughly with 50 ml of water. The mixture was applied to the leaves with an atomizer attached to a compressed air nozzle. The leaves were wrapped in plastic sacs and stored in an ISCO incubator (see above for conditions) for 24 or 48 hr at which time the plastic sacs were removed. The plants were incubated for another 24 hr and symptom development was then recorded on film.

PURIFICATION AND CHARACTERIZATION OF ACTIVATOR

Chromatography

Descending paper chromatography was carried out on Whatman No. 1 filter paper and upon water washed Whatman No. 541 filter paper in Solvent a: 1-butanol:acetic acid:water, 4:1:5 v/v.

Eastman Chromatogram Sheets (6061) coated with silica gel were employed for thin layer chromatography in the following solvent systems: (b) 96% EtOH:water, 7:3 v/v, (c) n-propanol:water, 7:3 v/v, (d) n-butanol:acetic acid:water, 8:2:2, v/v, (e) n-propanol:34% NH_4OH, 7:3 v/v, (f) 96% EtOH:34% NH_4OH, 7:3, v/v, (g) 2-butanone:pyridine:water: acetic acid, 70:15:15:2, v/v.

Spots were detected by spraying chromatograms with an alcoholic ninhydrin/3% acetic acid solution and heating at 100° for 10 min.

Paper strips coinciding with ninhydrin reactive spots were eluted and tested for biological activity, i.e., stimulation of helminthosporoside production.

Cation Exchange Chromatography

Dowex 50-x8, 200-400 mesh, was purchased from Sigma Chemical Co. Fine particles were removed by repeated decantation of a settled slurry of 50 g of resin. Two liters of 1 N HCl were passed through the resin bed, contained in a Buchner funnel, followed by 1 ℓ of H_2O. The process was repeated with 2 ℓ of 0.1 N HCl and 3 ℓ of water. The resin was stored under water in the cold until used.

High Voltage Paper Electrophoresis

High voltage paper electrophoresis was performed on a Shandon Model L 24 apparatus. Separations were achieved in a pyridine:acetic

acid:water buffer (1:0.5:10 v/v) pH 5.02, on water washed Whatman No. 3 filter paper, 22 x 56 cm, at 12V/cm for 2.5 hr.

Purification of Activator

Leaf wash method. Leaves of the susceptible clone 51 NG 97 were rinsed on both surfaces with deionized, distilled water applied with a squirt bottle. Roughly 3 ℓ wash water were collected in a large beaker.

Whatman No. 1 filter paper in a Buchner funnel removed debris from the wash water which was evaporated to dryness under vacuum at 45°.

The residue was taken up in 10 ml of water, and 5 ml loaded onto a 1.2 x 5 cm bed of Dowex-50 cation exchange resin stabilized with a 3-5 mm layer of sand. The columns were washed with 100 ml of water and developed with 500 ml of 4N NH_4OH followed by 100 ml of rinse water. This solution was evaporated to dryness under vacuum at 45°.

The residue was dissolved in 3 ml of water and subjected to three successive paper chromatographic separations in Solvent a. The runs were of 24, 36, and 24 hrs respectively. Following each run, the strip containing biological activity was eluted with water which was evaporated to dryness.

High voltage paper electrophoresis was conducted on a 0.1 ml solution of the residue from the final chromatographic separation. Nearly homogeneous activator eluted from the electrophaerogram was

further purified by chromatography on Whatman No. 541 filter paper in Solvent a for 10 hrs.

Leaf extraction method. Leaf tissue (1 g/5 ml H_2O) was extracted in an isothermal autoclave at 78° for 15 min. The extract was reduced to 0.1 the original volume under vacuum at 45°. Three volumes of acetone at -15° were added with stirring. The precipitate was removed by centrifugation at 20,000 x g for 15 min. Acetone was removed by flash evaporation and the remaining solution reduced to 15 ml. Portions of 5 ml each were loaded onto Dowex-50 cation exchange columns and treated as related previously.

The residue from the cation exchange columns was taken up in water and 3 ml portions loaded onto a Bio-Gel P-2 column (200-400 mesh), 1.5 x 80 cm. The column was eluted with water, collecting 1 ml fractions. Fractions 60-88 (Void Volume = 48), containing biological activity, were combined and evaporated to dryness under vacuum at 45°. The residue was taken up in water and subjected to the paper chromatographic and electrophoretic procedures reported above.

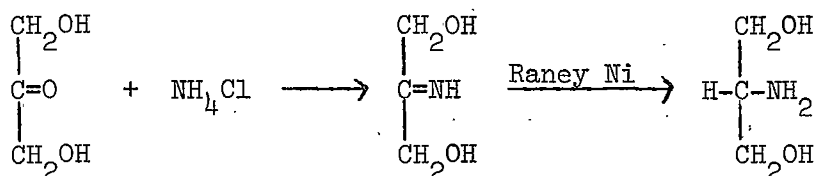
Instrumental Analysis

Melting points were determined on a Fischer-Johns Melting Point Apparatus. Infra red spectra were obtained on a Beckman Microspec Spectrophotometer using a KBr micropellet. A Model 25 Beckman Du Spectrophotometer provided ultraviolet spectra.

Low resolution mass spectrometry was conducted by Dr. Tom Krick, Department of Biochemistry, University of Minnesota, St. Paul, MN. Mass spectra were obtained on a LKB Model 9000 spectrometer with 20 eV on the filament and the probe heated to 80°.

Synthesis of 2-amino-1,3-propanediol

The following reaction describes the synthesis of 2-amino-1,3-propanediol:



Dihydroxyacetone, 0.2 g, NH_4Cl, 0.4 g, and Raney Nickel, 0.2 g, were refluxed in 100 ml of anhydrous methanol for 4 hrs at 65°. The reaction mixture was filtered through Whatman No. 1 filter paper and the methanol removed by evaporation under vacuum at 45°. The product, detected with the ninhydrin reagent following paper chromatography on Whatman No. 541 in Solvent a for 10 hrs, was eluted with water and tested for biological activity.

RESULTS

Attenuated variants of *H. sacchari* are those which cannot produce toxin in culture. Previous observation revealed that cane extract contained a substance that was capable of restoring or "activating" toxin synthesis in attenuated cultures.

This observation suggested that some insight into the problem of attenuation might be gained by obtaining attenuated variants of *H. sacchari*, purifying the activator from cane, and studying the interaction between the two.

Acquisition of Attenuated Variants of H. sacchari

Fig. 1 is a flow diagram describing the acquisition of attenuated strains of *H. sacchari*. A pathogenic strain of *H. sacchari* was successively subcultured on M1D medium until toxin could not be detected by bioassay.

Slants were grown for 5 days prior to transfer at which time a suspension of mycelium was used to inoculate both the succeeding agar slant and 500 ml Roux flasks containing M1D medium. The flasks were incubated for 18 days preceding bioassay.

Table 1 shows the number of transfers required for attenuation of *H. sacchari* in culture. Toxin production ceased after six transfers in two separate experiments. Symptoms were not quantitated, i.e.,

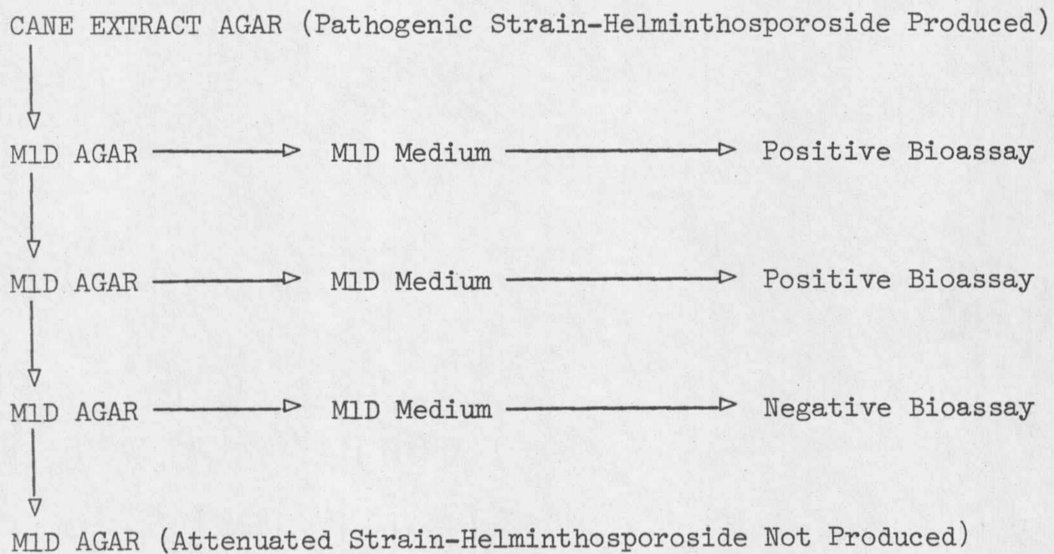


Fig. 1. Flow chart summarizing the procedure for detecting attenuation in culture. A pathogenic strain was successively subcultured on synthetic M1D medium until toxin could not be detected by bioassay. For procedural details see text.

Table 1. Number of transfers required for attenuation of pathogenic *H. sacchari* in culture. The (+) and (-) signs indicate the results of bioassays conducted on the MlD cultures at each step in the flow diagram in Fig. 1.

Experiment	Transfer Number							
Number	1	2	3	4	5	6	7	8
1	+	+	+	+	+	-	-	-
2	+	+	+	+	-	-	-	-

+ = Positive Bioassay-Symptom Production

- = Negative Bioassay-No symptom Production

runner length was not measured. Rather, the presence or absence of symptoms was considered indicative of the culture's status with respect to helminthosporoside production.

Purification of Activator

Once attenuated strains of *H. sacchari* were in hand there remained the task of isolating the activator. Fig. 2 is a flow chart illustrating the procedure used to isolate that compound from the leaf surface. An alternate procedure for purifying the activator from leaf extracts is described in Materials and Methods.

The biological activity of the activator at each step in purification was measured indirectly by quantitating the helminthosporoside produced in assay culture (Table 2). Runner length was converted to approximate toxin concentration (in nmols) using the equation derived by Strobel and Steiner (34).

Biological activity was present in several ninhydrin reactive areas of the paper chromatograms following separations I and II in Solvent a. Those areas with greatest activity were selected for further purification. R values of the selected area of biological activity for each of the four separations in Solvent a are given in Table 3. These values are relative to either phenylalanine or tryphtophan. The chromatogram from separation III in Solvent a and the electrophaerogram each contained a single area of biological