Alpha-galactoside-binding proteins from plant membranes : distribution, function, and relation to helminthosporoside-binding proteins of sugarcane
by Douglas Sherwood Kenfield

A thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Plant Pathology
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Abstract:
The critical, specific reaction during the molecular interplay between Helminthosporium sacchari and Saccharium of-ficinarum^ is the binding of helminthosporoside by a toxin receptor in the plasmalemmas of susceptible sugarcane. Because the toxin is an alpha-galactoside and other alpha-ga-lactosides compete with the toxin for receptor sites, the normal role of toxin receptors in plants may be as alpha-galactoside transporters. If so, similar proteins should exist elsewhere in the plant kingdom. A survey of nine plants revealed alpha-galactoside binding in trichloroace-tate extracts of cellular membranes from each. Raffinose, galactinol, and helminthosporoside binding occurred in extracts from susceptible sugarcane, mint, tobacco, wheat, barley, and beet. Extracts from resistant sugarcane, corn, and potato displayed raffinose and galactinol binding only.

Binding proteins from susceptible and resistant sugarcane, mint, and tobacco were purified via melibiose-Sepha-rose-6B affinity chromatography. All four proteins had a molecular weight of about 100,000 and displayed subunit species of about 50,000 and 14,000-20,000. Amino acid compositions were similar, but not identical. The protein from resistant sugarcane had less mobility than the other three during polyacrylamide gel electrophoresis,, At high protein concentrations (100 ug/ml), resistant cane and mint proteins did not bind helminthosporoside: susceptible sugarcane and tobacco proteins did, At low protein concentrations (4 ug/ml), all four proteins yielded Kd's of about 10-5M for raffinose, galactinol, and helminthosporoside.

The inverse correlation of binding activity with protein concentration was a logarithmic relationship for each protein-toxin combination. Presence of high molecular weight polymers was observed and the oligomer/polymer ratio was also found to be an inverse logarithmic function of protein concentration. In aqueous solutions, then, self-association of binding proteins is dependent on protein concentration and presence of ligand. Polymers are favored by high protein concentrations: oligomers are stablized by ligand.

Differential toxin binding by receptors from susceptible and resistant sugarcane required the presence of integral membrane components or exogenous galactolipids. Also, sensitivity to helminthosporoside increased in older portions of susceptible leaves. In vivo concentrations of receptors and composition of the membrane milieu likely modulate expression of the potential for toxin binding inherent in proteins from both susceptible and resistant sugarcane.

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ABSTRACT

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INTRODUCTION

Nearly every carbon atom in heterotrophs first exists as part of a sugar molecule in a plant. This fact marks the importance of expanding our knowledge of carbohydrate synthesis, translocation, and utilization if, in a melioristic sense, we, as plant scientists, hope to enhance the efficiency of food production.

Sucrose is by far the most common sugar in most plants. The alpha-galactosyl derivatives of sucrose, the raffinose family of carbohydrates, comprise the second-most prevalent group of sugars (40). Major enzymes which accomplish the biosynthesis of the most common alpha-galactosides have been identified (37). In certain plants (squash, white ash, and members of the Labiatae) galactinol, raffinose, and stachyose are known to be among the first products of photosynthesis where raffinose and stachyose predominate among translocated carbohydrates (8, 25, 53, 77). More recently, chloroplasts from peas have been shown to contain the enzymes necessary for the synthesis of myo-inositol, galactinol, and raffinose (28, 29).

Of interest now is an elaboration of the mechanisms by which these sugars are delivered out of the synthesizing cells and into the translocation system of the phloem. Few types of carbohydrates are translocated, the major ones

being sucrose, the raffinose family, and the alditols mannitol and sorbitol (77). Geiger has discussed in detail certain mechanisms which may account for this relative selectivity during phloem loading (19). One postulated mechanism entails mediation of solute transport by selective carrier systems in the membranes of phloem cells. Isolation and characterization of such selective carriers are the next logical projects, but before pursuing this course, consideration of a related mechanism of pathogenesis will be enlightening.

The eyespot disease of sugarcane is caused by Helminthosporium sacchari (van Breda de Haan) Butl. (= Drechslera sacchari (Butl.) Subram. & Jain). The pathogenicity of this fungus is dependent upon its ability to produce helminthosporoside, an alpha-galactoside with the proposed structure 2-hydroxycyclopropyl-alpha-D-galactopyranoside (57, Figure 1). Sensitivity to the toxin and susceptibility to the fungus are strongly correlated with the presence of a functional toxin-binding protein in the plasma membranes of host cells (61). After extraction with Triton X-100 from membranes of a susceptible clone of sugarcane (51NG97), the binding protein appears to be an oligomeric protein with a molecular weight of 49,000, containing four

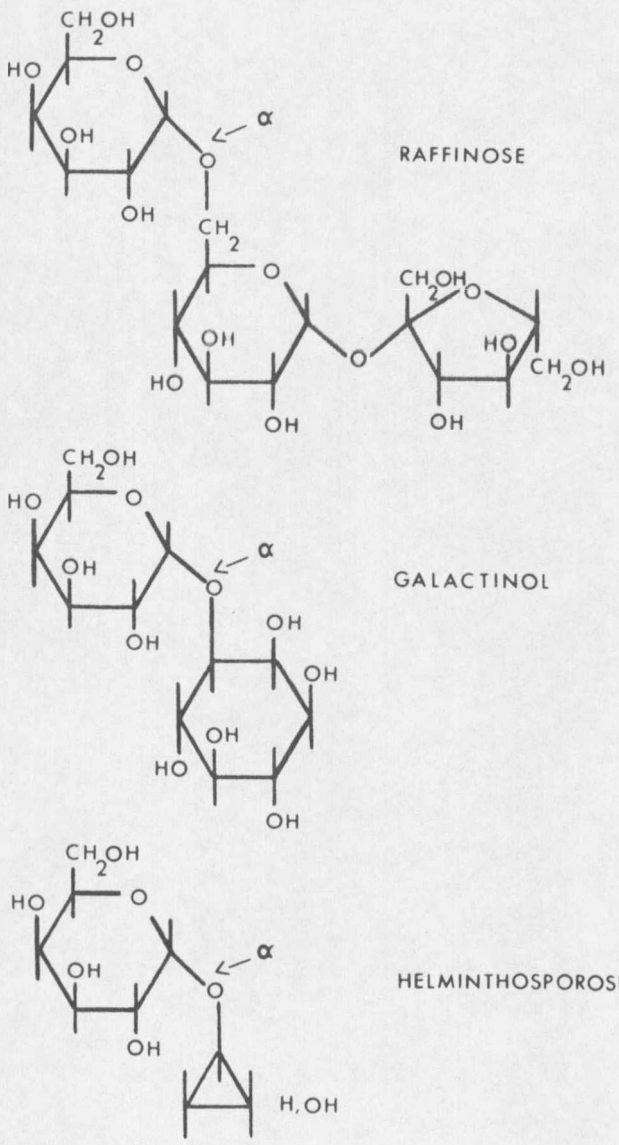

Figure 1. Illustration of raffinose and galactinol, common alpha-galactosides from higher plants, and helminthosporoside, a host-specific toxin produced by Helminthosporium sacchari (57). The aglycone of helminthosporoside is best represented as alpha-C_3H_5O at this time (Strobel-personal communication).

subunits and at least two binding sites (60). A resistant clone, H50-7209, contains a protein of the same molecular weight and subunit composition yet different from the 51NG97 protein in antigenicity, amino acid composition, and electrophoretic mobility. Further, freshly isolated H50-7209 protein lacks detectable toxin-binding activity just as protoplasts from resistant sugarcane do not exhibit a net uptake of helminthosporoside or raffinose (62). After three weeks in Triton X-100 or after treatment with sodium dodecyl sulfate (SDS), the detergent-solubilized protein from H50-7209 acquires the ability to bind toxin in vitro (61).

Based on these and other extensive observations, a molecular model of pathogenesis for the eyespot disease has been proposed (65). Briefly, the fungus infects susceptible hosts and produces at least one toxin, helminthosporoside. Helminthosporoside binds to sensitive proteins in plasmalemmas causing a perturbation of membrane constituents. Subsequently, ionic imbalance in the cells occurs; the cells swell and eventually burst. These events culminate in the leaves as water-soaked streaks which develop into reddish runners, characteristic symptoms of eyespot.

Many modes of resistance to H. sacchari are possible. The fungus's ability to produce helminthosporoside is controlled by the availability of either serinol or a "high-molecular-weight inducer," the syntheses of which occur in the host (5, 46). Potential hosts may prevent pathogenesis passively through a lack of or subminimal production of either or both of these inducers. In contrast to such passive resistance, sugarcane may elaborate inhibitors of helminthosporoside synthesis. To date, experimental evidence for this type of resistance is lacking. Alternatively, alpha-galactosides are known to protect leaves from the detrimental effects of the toxin (60), thus it is possible that sufficiently high levels of indigenous alpha-galactosides confer resistance. Another mode of resistance arises from the presence of toxin receptors which are modified such that, in situ, they express a diminished affinity for the toxin, even to the point of lacking toxin-binding ability altogether. This last example appears to be represented by the proteins from 51NG97 and H50-7209. Any, some, or all of these mechanisms of resistance could be operative in particular clones and confer to them high, intermediate, or low phenotypes relative to susceptibility to H. sacchari.

In the presence of helminthosporoside, however, the crucial step, the reaction which determines the possibility of subsequent processes leading to cellular death, is the binding of helminthosporoside to a toxin receptor in the plasma membrane. If binding is blocked, pathogenesis is precluded.

Certain ideas in the foregoing discussion are important in terms of relating this type of pathogenesis to carbohydrate transport. First, alpha-galactosides effectively inhibit activity of the toxin in leaves. Further, Triton X-100-solubilized proteins from 51NG97 exhibit similar K_d's for helminthosporoside, raffinose, and melibiose. Comparable kinetics are obtained for raffinose binding by isolated proteins and raffinose uptake by sugarcane protoplasts. Furthermore, pretreatment of protoplasts from susceptible sugarcane with helminthosporoside blocks uptake of raffinose. These results support the hypothesis that the helminthosporoside-binding protein is part of an alpha-galactoside transport system normally present in sugarcane cells (63). As noted in the original presentation of this hypothesis, an anomaly occurs in H50-7209, resistant sugarcane, whose receptor lacks detectable raffinose- and toxin-binding activities. If alpha-galactoside transport is a

normal process in the translocation of carbon skeletons in sugarcane, how does H50-7209 compensate for the apparent lack of this process? Abstractly related to this question, consideration of the probability of alpha-galactoside transport in sugarcane and the prevalence of alpha-galactosides in the plant kingdom leads to the expectation that toxin-binding (= alpha-galactoside-binding) proteins exist elsewhere among plants. If such proteins do exist, what additional information could be gleaned from them in terms of both the toxicity of helminthosporoside and the mechanisms of alpha-galactoside transport? Answers to the first two of these questions are apparent upon evaluation of the results of a survey of the plant kingdom for alpha-galactoside-binding activity (33).

Membrane preparations from numerous, diverse plants were assayed for abilities to bind galactinol, raffinose, and helminthosporoside (Figure 1). Crude membrane proteins were prepared as outlined by Strobel (60) with one exception. Trichloroacetate, a chaotrope, was used instead of Triton X-100 to release the proteins from the membranes. All of the plants possessed galactinol- and raffinose-binding activities. All but three displayed a detectable toxin-binding activity.

Apparently, alpha-galactoside-binding activity in general and toxin-binding activity in particular are prevalent among plants. Also, resistant sugarcane does contain alpha-galactoside-binding proteins, the activity of which is somehow masked in detergent extracts and protoplasts. Ligand specificity in crude extracts is exampled by the analogous activities of corn, potato, and H50-7209 proteins: they bind raffinose and galactinol but not helminthosporoside. This apparent specificity looms in view of the binding data reported in Chapter 3.

Intriguing also is the knowledge that toxin-binding activity exists in most of the plants tested, yet only susceptible sugarcane develop symptoms upon exposure to the toxin. Further, tobacco protoplasts are not sensitive to the toxin even though membrane extracts from this plant possess toxin-binding activity. Upon treatment with purified toxin-binding protein from 51NG97, however, tobacco as well as H50-7209 protoplasts acquire sensitivity to helminthosporoside and burst in its presence (64). Two possible explanations exist for the situation in tobacco and the other plants which bind yet are not sensitive to the toxin. The binding sites may be unavailable to extracellular toxin

because of the inaccessibility of the protein or the presence in the membrane of effectors which inhibit binding. Alternatively, binding may occur but subsequent events essential for cellular demise may be precluded. Once toxin binding has occurred in susceptible sugarcane, a heat-sensitive reaction(s) must follow or the cell remains intact. Sugarcane clones which initially are susceptible to H. sacchari and sensitive to helminthosporoside become resistant and insensitive after exposure to warm temperatures (30-35C), treatment with hot water (30-50C), preincubation in an N_2 atmosphere, or preincubation with protein-synthesis inhibitors. Although hot-water-treated leaves could revert to a sensitive condition, cold temperatures (5-15C), N_2, or protein-synthesis inhibors prevent recovery of sensitivity (12). Concomitant with the temporary, heat-induced resistance in usually susceptible sugarcane is a 50% reduction in the activity of membrane ATPase but no loss of helminthosporoside-binding activity. Recovery of sensitivity and restoration of ATPase activity occur within 24 h. Treatment of plasma-membrane-enriched suspensions with helminthosporoside causes a 30% activation of ATPase activity in susceptible sugarcane preparations. No activation is detected in analogous preparations from normally resistant

sugarcane, heat-induced resistant sugarcane, or mint. Arrhenius plots of toxin-induced symptoms and membrane ATPase activity reveal a sharp change in slope at 32C for each. Finally, inhibitors of membrane ATPase activity protect susceptible leaves from the effects of helminthosporoside. Furthermore, protoplasts of susceptible sugarcane are insensitive to toxin in a K^+, Mg^{++}-deficient medium. Sensitivity is restored upon addition of these ions (66). Thus, one heat-sensitive reaction subsequent to toxin binding and essential for pathogenesis appears to be the activation of a plasma-membrane-bound (K^+, Mg^{++})-ATPase. Whether this activation is directly or indirectly associated with toxin binding or whether stimulation of ATPase activity is the only reaction pursuant to toxin binding is unknown. Regardless of the exact nature of the reactions following binding, they are mediated via the initial interaction of helminthosporoside with the toxin receptor. The receptor from susceptible sugarcane must possess certain unique properties which cause it somehow to perturb the plasma membrane upon binding helminthosporoside. Such a perturbation occurs even in heterologous protoplasts, further evidence that the binding protein from susceptible sugarcane is the potentiator of this mechanism of pathogenesis.

This dissertation presents the isolation, purification, and characterization of the alpha-galactoside-binding proteins from susceptible sugarcane, resistant sugarcane, mint, and tobacco. One aim of this comparative biochemical analysis is to illustrate the uniqueness of the 51NG97 protein. An additional goal is to make feasible the study of these and similar carbohydrate-binding proteins in plants.

The dissertation is divided into three major topics: the comparative biochemistry of the four proteins, analysis of the *in vitro* binding activity, and an appraisal of the role of sugarcane proteins in the eyespot disease. Following these chapters will be a discussion of the involvement of binding proteins in such phenomena as disease resistance, carbohydrate transport, and cellular ageing.

MATERIALS AND METHODS

Isolation

Unless otherwise specified, the following isolation scheme was employed to obtain purified binding proteins. Crude membrane preparations were obtained as described by Strobel (60). These preparations were either lyophilized or homogenized immediately in Tris-HCl, 50 mM, pH 7.0 containing 1.0 M sodium trichloroacetate. A ratio of 20 ml of buffer per 100 g fresh weight of leaves was used. After one hour at 4C, the homogenate was centrifuged at 48,000g for 15 minutes at 4C. The supernatant was passed over a Sephadex LH-20 column (0.9 x 20 cm), eluting with Tris buffer which lacked the trichloroacetate. Five A_{265}-absorbing peaks were observed plus a large amount of colored material which passed from the column in the 70% ethanol rinse following each run. Only the material which eluted near the void volume contained measurable binding activity.

Fractions at or near the void volume were collected and diluted with three volumes of buffer then passed over a melibiose-Sepharose-6B affinity column having a bed volume of 160 ml (17). The column was washed with Tris buffer until no uv-absorbing material was detectable. Ten ml of 1.0 M melibiose was then added followed by Tris buffer. One column volume was collected, concentrated *in vacuo* at 30C to

approximately 10 ml, then desalted on a Sephadex G-25 column (2.5 x 50 cm). The fractions near the void volume were collected, concentrated in vacuo at 30C to 20 ml, then dialyzed under vacuum against either water or Tris buffer. The dialysate was collected, then the dialysis bag was rinsed with Tris-HCl, 1.0 M, pH 7.0. The dialysate and rinse material were combined, then desalted twice on the G-25 column. The excluded material was again concentrated in vacuo at 30C to 1 ml at which time it was either assayed directly or frozen and lyophilized (Figure 2).

Protein quantitation

Purified protein was quantitated based on the difference in absorption at 215 and 225 nm using a Beckman Model 25 Spectrophotometer (71). A standard curve was developed with bovine serum albumin. Determinations made by this nondestructive method of quantitation compared well with values obtained using the methods of Lowry, et al. (38), Schaffner and Weismann (51), or Bio-Rad (9). Because residual trichloroacetate strongly interfered with the differential absorption method, crude protein preparations were quantitated by the Lowry or Bio-Rad procedures.

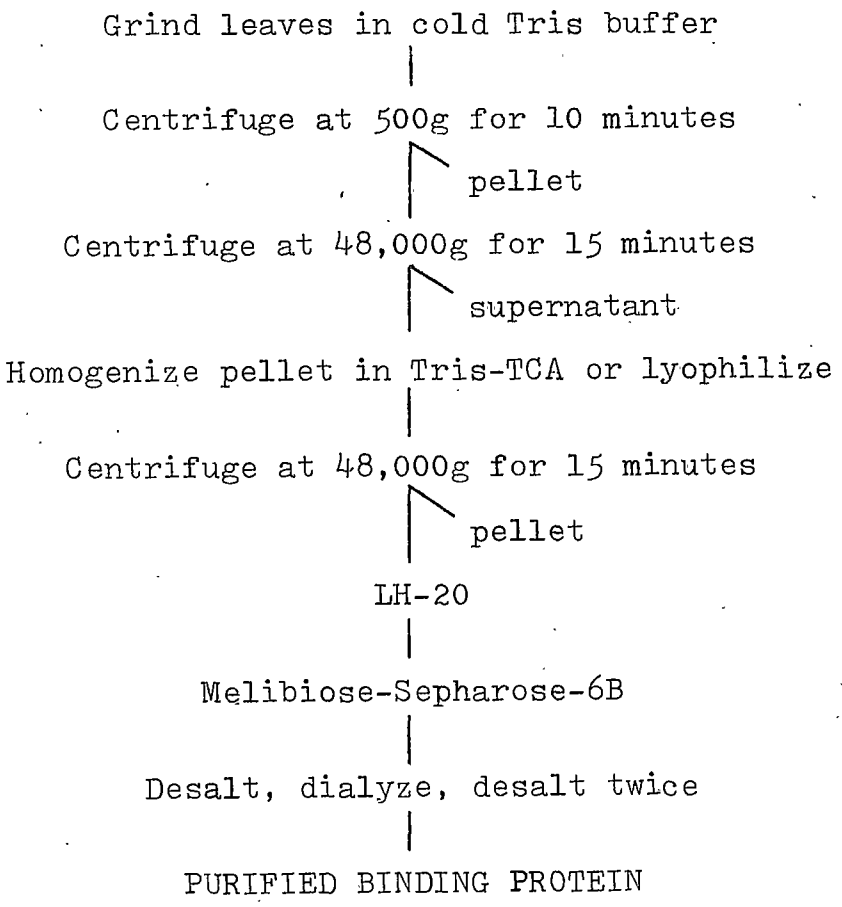

Figure 2. Flow chart illustrating the isolation of alpha-galactoside-binding proteins from plant leaves.

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) on 7.5% gels was performed according to Ornstein and Davis (15). Coomassie blue stained the proteins poorly, but attempts to stain with Amido Schwarz, Stains-all (Sigma), or oil red O were unsuccessful.

Fluorescamine staining

Fluorescamine staining of the proteins was accomplished by dissolving 15-20 ug of lyophilized binding protein in 50 ul of sodium borate, 50 mM, pH 8.1, containing glycerol at a final concentration of 50% (v/v). Fluorescamine (Sigma) was then diluted with distilled acetone to a concentration of 3 mg/ml and added quickly to the protein preparation while mixing vigorously. The mixture was then allowed to stand at room temperature for 10 minutes prior to electrophoresis (49).

Molecular weight estimations

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to estimate molecular weights of the purified binding proteins (75). Also, molecular weight estimates via gel filtration were obtained using columns of Bio-Gel P-100 and P-200, Sephadex A1.5, and

Sepharose 6B. The elution buffer was Tris-HCl, 10 mM, pH 7.0. Standard proteins used were those in the Combithek kit (Boehringer-Mannheim, Figure 4) and Corynebacterium insidiosum toxin which has a molecular weight of 5×10^6 (48). Column effluents were monitored with a Gilson UV-RP flowmeter equipped with an A_{265} interference filter.

Amino acid analyses

Amino acid analyses of 50 ug aliquots of purified binding proteins were performed by the aaa Laboratory, Seattle, WA. Proteins were hydrolyzed for 24 h at 110C in 6N HCl, then subjected to ion exchange chromatography on a Durrum Analyzer, Model D-500. Yields of threonine and serine were augmented 5% and 10%, respectively, to account for loss during hydrolysis. Tryptophan and cystine were not quantitated.

Ligand affinities

Ligand affinities were measured via equilibrium dialysis using a Kontron Diapak dialysis unit (60). The dialysis membrane, Spectra/Por 1, had a molecular weight exclusion limit of 8000 (Spectrum Inc.). Equilibration of raffinose required 4 h. Dialysis time for measuring the ligand affinities was 8 h. Dialysis chambers were immersed

in a 30C water bath and rotated at 24 rpm. At the end of the dialysis period, the contents of each half cell (0.5 ml) was placed in 5 ml of Aquasol (Nuclear Chicago) and counted for 100 minutes in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3320. The total counts over this time period ranged from 10,000 to 30,000 cpm. At the 90% confidence level (1.6 standard deviations), the % deviation was 1.6 to 0.9 (3). Counts were corrected to dpm using the channel ratios method for quench correction.

Preparation of radio-labeled ligands

^{14}C-Labeled ligands were variously prepared. Galactinol was labeled using a galactosyl transferase isolated from pea seeds to combine myo-inositol with ^{14}C-UDP-galactose (18). Radioactive raffinose was prepared by a transgalactosidation between ^{14}C-sucrose and raffinose (41). Galactinol and raffinose were also radio-labeled by incubating mint plants for 2 hours with $^{14}CO_2$ and isolating the sugars (53). Authentic galactinol was generously supplied by C. E. Ballou.

^{14}C-Helminthosporoside was purified from stationary phase cultures of Helminthosporium sacchari incubated with ^{14}C-sucrose 4 days at 24C (57).

The specific radioactivities of the alpha-galactosides were:

Ligand	uCi/umole	kBq/umole
raffinose	3.87×10^{-1}	1.43×10^{1}
galactinol	2.58×10^{-3}	9.55×10^{-2}
helminthosporoside	4.59×10^{-3}	1.70×10^{-1}

Plant material

Plants used in this study include sugarcane clones 51NG97 and H50-7209 (susceptible and resistant to H. sacchari respectively), mint (Mentha spicata), tobacco (Nicotiana tabacum 'White Burley'), and napier grass (Pennisetum purpureum). These plants were maintained in the university greenhouse with no auxiliary lighting. They were potted in coarse, sandy loam and watered with a fertilizer solution consisting of 20:20:20 NPK, 0.5 lb/gal, diluted 1:100 with tap water. Temperature was maintained between 18-24C.

Cultures of H. sacchari were originally obtained from Dr. Gary Steiner, Hawaiian Sugar Planters Association. The fungus was maintained on cane agar slants prepared according to Pinkerton (47).

Testing susceptibility and sensitivity of plants

Susceptibility of the study plants was tested by

spraying the leaves with aqueous suspensions of spores and mycelia of H. sacchari. The misted plants were enclosed in plastic bags for two days after which the bags were removed. Plants were then maintained in a growth chamber for six weeks with the following daily regimen:

Period	Duration	Temperature	Relative Humidity
interim	2 h	70C	80%
day	8 h	80C	60%
interim	2 h	70C	80%
night	12 h	60C	90%

During the day and interim periods, the light intensity was 3×10^4 ergs cm^{-2} sec^{-1}.

Plants were assayed for sensitivity to helminthosporoside by the technique of Steiner and Strobel (57). Droplets of aqueous toxin (2 ul, 4.5 mM) were placed over puncture sites in the leaves. The leaves were placed in a humidity chamber at room temperature and visually monitored each day for six days.

Chemicals

2-Chloroethanol (Aldrich) was distilled before use. Monogalactosyl diglyceride and digalactosyl diglyceride (Applied Sciences, Inc.) were originally isolated from soybean. The remaining chemicals were obtained from Sigma Chemical Company.