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ISOLATION OF HYPOXYLON MAMMATUM TOXINS

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ISOLATION OF *HYPOXYLON MAMMATUM* TOXINS AND EFFECTS ON *POPULUS TREMULOIDES*

By

Bruce Allen Stermer

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ABSTRACT

ISOLATION OF *HYPOXYLON MAMMATUM* TOXINS
AND EFFECTS ON *POPULUS TREMULOIDES*

By

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Clones of *P. tremuloides* varied in sensitivity to toxins from *H. mammatum*, as shown by effects on leaves and young stems. A leaf assay, sensitive enough to detect toxin in samples containing 10 ng dry weight, was based on uptake of toxin solution by cut petioles. Leaves from a sensitive clone showed black necrosis by 5 h after exposure; another clone tolerated 500-fold more toxin with no obvious damage. Sensitivity of stems was correlated with sensitivity of leaves. Leaves of the sensitive clone responded within 1 h by increased respiration (+36%); O_2 uptake by the tolerant clone was not affected. Toxin caused an increase (+500 to 600%) in leakage of electrolytes from tissues of the sensitive, but not the tolerant, clone, beginning 6 h after exposure. Toxin was partially purified by use of Bio-Gel P-2 and silicic acid columns. Several host-specific toxic components were recovered. These and other data suggest that the toxins are involved in development of Hypoxylon canker.

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INTRODUCTION

Hypoxylon canker caused by Hypoxylon mammatum (Wahl.) Miller is one of the most important diseases of quaking aspen, Populus tremuloides Michx., in North America. It occurs throughout most of the range of quaking aspen in the United States and Canada. In Michigan, Wisconsin, and Minnesota, more than 12% of the live aspen are currently infected with Hypoxylon canker, resulting in economic losses for this valuable source of fiber in the millions of dollars per year (28). An estimated 300 million ft^3 of aspen wood is lost each year in the Lake States as the result of Hypoxylon canker; accumulation of these yearly losses until harvest means the annual growth and utilization of aspen could be doubled if Hypoxylon canker were eliminated (28). However, no effective control measures for Hypoxylon canker are known.

Schipper (40) reported that a toxin produced by H. mammatum was necessary for the infection of aspen by the fungus. Other investigators (13,18) have attempted to screen for resistance in aspen to Hypoxylon canker by selecting trees tolerant of the toxin from H. mammatum. The purpose of my study was to further purify the toxin, to learn more about the character of the toxin, and to examine some of the effects of the toxin on aspen tissues.

LITERATURE REVIEW

Hypoxylon canker was first found in 1920 in Essex County, New York, and Oakland County, Michigan, and described by Povah (32) in 1924. However, the fungus itself was described by Klotsch (26) in 1833 as Sphaeria pruinata. Later in 1883 Cooke (14) transferred it to the genus Hypoxylon and described the fungus as H. pruinatum (Klot.) Cooke. Recently, Miller (29) has combined H. pruinatum with H. mammatum (Wahl.) Miller. Koch's postulates were completed for Hypoxylon canker by Bier (9) in 1940; the general characteristics of the disease have been reviewed by Gruenhagen (20).

Host Range

The most common host of H. mammatum is Populus tremuloides. Other hosts in decreasing order of susceptibility are P. grandidentata Michx., P. balsamifera L., and P. trichocarpa Torr & Grey (21). Naturally occurring cankers have also been described on P. tremula L. (3), and on Acer rubrum L. and A. saccharum Marsh. (8). Miller reported H. mammatum on wood of Acer, Alnus, Betula, Carpinus, Fagus, Picea, Populus, Pyrus, Salix, Sorbus, and Ulmus; however, he does not discuss pathogenicity to these trees (29).

Attempts at inducing Hypoxylon cankers by artificial inoculation have given various results. Generally, efforts have been unsuccessful in producing cankers with H. mammatum by inoculating aspen buds, leaves,

petioles, or leaf axils with mycelia or with suspensions of ascospores or conidia (9,20,42,43). However, cankers developed readily on stems and branches inoculated with mycelia (9,20,42,43). H. mammatum has a wider host range on excised branches; cankers are produced on cuttings of P. deltoides, P. deltoides x P. balsamifera (7), Acer macrophyllum Pursch., and Salix sp. (10).

Mode of Infection

Transmission and the mode of infection for H. mammatum is uncertain. Researchers have been unable to infect aspen by inoculating the unwounded periderm and agree that the fungus is a wound parasite on branches and trunks (9,20). The type of wounds which serve as infection courts is not clear, but insect injuries are commonly associated with cankers. Anderson et al. (2) found that out of 169 natural cankers in an aspen plantation in Minnesota, 95% originated in galls induced by the insect Saperda inornata, although only 1% of the total galls were infected. Similar observations in Michigan showed that an important relationship exists between Hypoxylon canker and insects of aspen (19). More studies of the aspen-insect-fungus interaction are needed before the role of insects in Hypoxylon canker is understood.

The major inoculum for natural infections is thought to be ascospores (7), but efforts to infect with the spores have been negative (9,42). Ascospores are discharged from stromata throughout the year following precipitation, either rain or snow, even when the ambient temperature is below 0 C (47). Hypoxylon cankers bearing perithecia are present in most, if not all quaking aspen stands within the range of the disease, and cankers are obtained easily by wound inoculation with mycelia derived directly from ascospores. Therefore, Berbee and Rogers

(7) suggested that certain requirements are necessary for gerimination of ascospores or for the initial mycelial growth and that these requirements must be met for producing cankers. Reports of ascospore germination in the sapwood (1) and the xylem (5) of aspen indicates that infection by H. mammatum depends on conditions for mycelial establishment and not on ascospore germination.

Bagga and Smalley (5) consistently produced cankers on aspen inoculated with ascospores when cell-free fungal extracts from H. mammatum cultures were applied topically over the inoculated wounds. No cankers developed when ascospores were inoculated with 1% glucose, 2% malt extract, or water. The materials produced in culture were phytotoxic and caused extensive bark necrosis when applied to P. tremuloides stems. Bagga and Smalley concluded that a heat-resistant, water-soluble product from the culture filtrate was necessary for the production of cankers by ascospores.

In further studies, Schipper (40) found that an avirulent or a virulent isolate of H. mammatum successfully infected aspen when exogenous "mammatoxin" from culture filtrates was applied to the inoculated wounds; there were no successful infections by either isolate when toxin was withheld. Schipper noted that infection of aspen by H. mammatum has been successful only when mycelium growing on agar or grain was introduced into wounds, and that both agar and grain upon which H. mammatum has grown contains measurable amounts of the toxin. He suggested that successful natural infection occurred only when mycelium and toxin were introduced into wounds on aspen concurrently, perhaps by a vector carrying a piece of cankered host tissue into a wound on a healthy aspen tree.

Histology of Pathogenesis

H. mammatum grows primarily in the sapwood of infected trees and invades the bark from within (35). The fungus apparently invades only dead bark, presumably because aspen bark contains fungitoxic compounds (41). The pathogen has been isolated from discolored wood up to 5 cm beyond the visible canker limit, and under sound bark of *P. tremuloides* (25). Macroscopic and microscopic examination of inoculated wounds revealed that bark collapse preceded the hyphae of *H. mammatum* in the bark (38). The toxin found in culture filtrates of the fungus may be responsible for the bark necrosis seen in Hypoxylon cankers (41). The pathogen was isolated by Gruenhagen (20) from wood up to 0.8 cm beneath the cambium. Bagga (4) isolated *H. mammatum* from a naturally infected stem (9 cm dbh) throughout the entire area of discolored wood, up to the pith.

Effects of Environmental Factors on Development of Hypoxylon Canker

The effect of the environment on Hypoxylon canker has been studied extensively by many workers. Any factor which contributed to moisture stress in the host increased susceptibility to *H. mammatum* (6). Relative humidities higher than 50% and soils with high water-holding capacities were unfavorable for canker development. Non-fertilized plants were more susceptible than plants fertilized regularly.

A model accounting for 92% of the variation in canker incidence was constructed by Bruck and Manion (12) using 56 measured parameters. Highly significant negative correlations were observed between canker frequency and soil moisture. They summarized their results by stating that physical and chemical parameters of the soil which relate most directly to moisture were highly correlated with Hypoxylon canker incidence.

Host Resistance to *H. mammatum*

Factors responsible for resistance to Hypoxylon canker have not been established. Berbee and Rogers (7) postulated that rapid formation of callus around wounds was important for resistance. They observed rapid production of large amounts of wound callus in species of *Populus* resistant to *H. mammatum* infection, but little callus in susceptible species. Constitutent bark materials may also be involved in resistance to infection (17). Aqueous extracts from the outer bark of *P. tremuloides* stimulated ascospore germination of *H. mammatum*, while extracts from the underlying green layer inhibited their germination. Hubbes (23) reported that extracts of aspen bark strongly inhibited the fungus in culture, and attributed the inhibition to pyrocatechol, two glycosides, and possibly other substances in the bark.

Recent work has shown that phytoalexins are produced when *H. mammatum* is placed on debarked aspen stem disks (15,16). The significance of this is questionable; although mycelial plugs of *H. mammatum* caused production of phytoalexins, the phytoalexins were not capable of inhibiting mycelial growth of *H. mammatum*. Germination of the ascospores of *H. mammatum* were inhibited markedly by the aspen phytoalexins, but the spores did not induce the stem disks to produce phytoalexins. Other mechanisms of resistance to Hypoxylon canker that have been suggested include microbial competition (11) and rapid death of infected branches (46).

Genetic control of resistance to *H. mammatum* in aspen is complex. In inoculation tests of aspen species and their hybrids there was a lack of dominance for resistance or susceptibility to infection (27). The hybrids were intermediate to their parents and varied in susceptibility to the

fungus. In contrast, a study by Berbee and Rogers (7) suggested that susceptibility of aspen to Hypoxylon canker was dominant. Overall, evidence indiciates that the reaction of aspen to Hypoxylon canker is controlled by many genes (27,45,46).

The H. mammatum Toxin

In 1964 Hubbes was the first to report that H. mammatum produced substances that were toxic to P. tremuloides tissues, and that the diffusible substances prevented wound callus formation (24,25). In order to have successful initiation of canker development with H. mammatum ascospores, application of toxic materials produced in culture by the fungus was necessary (5). The fungal metabolite was most toxic on the tree species most susceptible to Hypoxylon canker, P. tremuloides, and much less toxic on other Populus species (38). The fungal metabolite is a host-specific toxin that causes bark collapse around aspen stem wounds identical with that caused by H. mammatum infection. Cell-free extracts of H. mammatum increased the activity of peroxidase enzymes in treated aspen seedlings (39).

The necrotic effect of the toxin on tissues of P. tremuloides is the basis of a bioassay. Schipper (38,41) placed fully expanded young aspen leaves in 27 ml vials that contained enough of the test solution to immerse the cut end of the petiole. The leaves were examined for necrosis after 16 to 96 hours of exposure. In another leaf bioassay, excised leaves were punctured with a 16-gauge hypodermic needle and a 20 µl drop of test solution was immediately placed on the puncture (18). Leaves maintained in humid chambers developed a discrete black necrotic spot which radiated from the point of toxin application. Aspen stem bioassays have been tested, but they have not proven to be useful (41).

Only P. tremuloides was highly sensitive to the toxin from H. mammatum, as shown by leaf bioassays of numerous plants in 24 different genera. French (18) reported that clones of P. tremuloides varied greatly in sensitivity of the toxin; many clones growing in the field were significantly tolerant of the toxin. French was not able to correlate tolerance to the toxin with resistance to infection by H. mammatum.

All isolates of the fungus tested by French produced toxin in culture, but the non-pathogenic isolates or those with low virulence produced smaller amounts of toxin (18). The fungus produced the toxic metabolites under all tested conditions which allowed growth of the fungus. The toxin has been isolated from Hypoxylon cankers (41).

The toxin produced in culture by H. mammatum was partially purified and was described as a heat-stable, non-protein compound with a mol wt of 700 to 1,100 (18, 41). The toxin was highly soluble in acetone, alcohols, and water, but was insoluble in chloroform, ether, or n-hexane. As many as 7 toxic forms were detected when the toxin was subjected to thin layer or silicic acid column chromatography (41).

MATERIALS AND METHODS

Plant Material

Young aspen trees were obtained from root cuttings of naturally occurring clones of *P. tremuloides* located near East Lansing, Michigan. The clones were identified by French (18) as highly sensitive to *H. mammatum* toxin (clone 5), highly tolerant to the toxin (clone 2), or intermediate in reaction to the toxin (clone 3). Subsurface runner roots 2.5 cm or less in diameter were collected, scrubbed in soap and water, cut into sections 10 to 15 cm long, and planted in a 1:1 sand-vermiculite mixture. Root sprouts were excised from root sections when 3 to 5 cm tall, and planted in the same mixture in flats covered with saran wrap. Rooted sprouts were then transplanted into 4 inch pots. When the young trees were 75 to 100 cm tall they were trimmed to encourage branching and planted in 10 inch pots. The plants were potted in a soil-less mix (sphagnum peat moss, vermiculite, and perlite) and fertilized biweekly with 20-19-18 soluble fertilizer. Spraying was done as needed for mite control with Plictran or Vendex at a rate of 3/4 teaspoon per gallon. A photoperiod of at least 16 h was maintained with supplemental illumination.

Fungal Material

The culture of *H. mammatum* used for toxin production was a single spore isolate previously described by French as isolate RL5A-7 (18).

Production of Toxin

One liter Roux bottles containing 200 ml of a modified Fries medium (33) were inoculated with 1 ml of blended mycelium. The bottles were kept stationary at 24 C under low light. After 2 to 7 weeks the culture fluid was harvested by filtration through 2 layers of cheesecloth, followed by filtration through a layer of miracloth in a Buchner funnel. The crude filtrate was concentrated under reduced pressure at 30 to 40 C to approximately 10% of its original volume. An equal volume of methanol was added and the solution was stored overnight at 4 C. Precipitates were removed by filtration through Whatman GF/C filter paper and the solution reduced to approximately 2.5% of the original volume. The concentrated, deproteinized, aqueous culture filtrate was partitioned 3 times against equal volumes of chloroform and then 3 times against equal volumes of water-saturated n-butanol. The butanol extracts, which contained most of the toxic activity, were combined, reduced to near dryness, and resuspended in 300 ml of distilled water. Activated charcoal (HCl-washed, from Sigma Chemical Co.) was added (4 to 5 g per liter of original filtrate) and the slurry was stirred overnight at 4 C. The charcoal slurry was centrifuged, the supernatant discarded, and the pellet washed by resuspending in water and recentrifuging. Next the toxin was desorbed from the charcoal by 3 washings with water-saturated n-butanol. The butanol fractions were combined, reduced to near dryness and resuspended in a minimum amount of water. The concentrated toxin preparation was then chromatographed through a Bio-Gel P-2 or a Sephadex G-15 column (1.5 x 90 cm) with water as the solvent, at a flow rate of 0.1 ml per min. The fractions from the column were assayed for toxicity and the toxic fractions were combined. This was the partially purified toxin used in experiments.

Bioassay

Solutions were assayed for toxin using a leaf puncture method similar to that used by French (18). Excised leaves were punctured with a needle, and a 10 μl drop of test solution was placed immediately on the puncture. The petioles of the leaves were immersed in water in vials which were kept in humid chambers. The diameters of the resultant lesions were measured at 24 or 48 h after treatment. All assays were replicated on leaves of the toxin-sensitive clone 5 and the toxin-tolerant clone 2, to determine host-specificity.

Thin Layer Chromatography

Both Analtech Silica Gel GF and Merck Silica Gel 60 plates were used. Because the Merck plates had a more durable surface and also gave better resolution, they were used in all the thin layer chromatography (TLC). Substances on the plates were assayed either for toxin or treated for observation. The most common method was to first spray the plate with a fine mist of concentrated H_2SO_4, observe the plate under longwave UV light for fluorescent materials, then heat the plate in a 90 to 100 C oven for 20 min and observe the charred materials. For the bioassay, 0.5 or 1.0 cm^2 areas of gel were separately scraped into test tubes, suspended in 0.5 ml of distilled water, shaken thoroughly and the solution assayed when the gel had settled. Typically, duplicate samples were chromatographed on the same plate so that after assaying for toxin the corresponding areas could be observed. All TLC plates were prewashed with 95% ethanol.

High Pressure Liquid Chromatography

The machine used was a Varian LC 5060 HPLC with a Whatman Co:Pell ODS precolumn and a Varian 4.1 x 300 mm MCH-10 (C_{18} reverse phase, 10 μm particles) column. A 100 μl aqueous toxin sample was injected into the machine and eluted with gradients of increasing ethanol concentration in water. A UV detector monitored absorption at 280 nm at the outlet of the column. Fractions were collected, the ethanol was removed, and then assayed for toxin after equalizing fraction volumes.

Absorbance Spectroscopy

Continuous visible-UV absorbance spectra from 800 to 200 nm were produced with a Cary 15 recording spectrophotometer (1 cm cell, scale 0 to 1.0 O.D.).

High Voltage Paper Electrophoresis

Toxin samples were subjected to electrophoresis on a 115 cm long sheet of Whatman #4 paper in a Savant high voltage paper electrophoresis apparatus. The buffer used was pyridine/acetic acid/water (25:1:225) pH 6.5 (30). The paper was run in kerosene electrophoretic coolant at 7000 v and 100 ma for 1 h. The toxin was detected by cutting the paper into sections, eluting the pieces with water, and assaying the water extract for host-specific toxicity. A mixture of acidic, neutral, and basic amino acids were also run as standards; they were detected by dipping the paper in a ninhydrin solution and heating after the toxin-containing segments had been removed.

Ion Exchange

Columns used for ion exchange chromatograhy were 5 3/4 inch glass Pasteur pipets. A glass wool plug was used in the narrow end to support the exchanger bed, and a serum bottle cap with a length of teflon tubing through its center was used to seal the top of the column. The exchangers were slurry packed and washed with 1N HCl and 1N NaOH. A 0.2 to 0.5 ml sample of toxin was applied to the top of the column bed for elution.

Warburg Manometry

Oxygen consumption was measured by a Warburg apparatus. Excised leaves were cut into 1.0 cm disks, enclosed in cheesecloth bags, weighted down and vacuum infiltrated in the appropriate solution for 15 min. The treated disks were rinsed and randomized, then approximately 0.1 g (10 to 15 disks) were placed in a Warburg flask containing a paper wick and 20% KOH in the center well. After attaching the flasks to the apparatus, they were equilibrated in the 30 C water bath, and the O_2 uptake was detected manometrically over the next few hours (44). The experiments were carried out in darkness.

Electrolyte Leakage

Leaves used in leakage experiments were allowed to take up a 10 µl treatment solution that was applied to the petiole end. The cut end of the petioles were then placed in vials containing water and permitted to transpire for 30 min. One cm disks were cut from the leaves, rinsed in distilled water, and randomized. Approximately 0.1 g of the disks were placed in liquid scintillation vials and suspended in 2.0 ml of distilled water. Electrolyte leakage of the leaf disks was determined by measuring

the conductivity of the ambient solution with an Electromark conductivity meter and a pipet-type electrode (K=1.0) (37).

RESULTS

Toxin Isolation Procedures

Early stages in toxin purification. The toxin was very soluble in methanol, ethanol, n-butanol, acetone, and water. When partially purified toxin or toxin in crude culture filtrates was partitioned against butanol, more than half of the toxin was found in the butanol fraction. Three successive partitionings extracted almost all the toxin from aqueous solutions. Contrary to earlier reports (18,41), experiments in this study showed that low amounts of toxic activity were extracted by partitioning with non-polar solvents.

The toxin was a relatively stable substance. Autoclaving for 20 min did not decrease the toxicity or affect the specificity of the toxin. When a 50% methanol solution of toxin was left on a countertop and exposed to bright diffuse light for 3 months, no changes in its host-specific activity was observed.

Toxin in aqueous solution was bound to activated charcoal; the toxin was then desorbed from charcoal with alcohols. n-Butanol always removed toxin bound to charcoal, but results with ethanol varied. In experiments with 95, 50, and 10% ethanol, toxin was inconsistently desorbed; on one occasion, all concentrations of ethanol removed much of the toxin, on another occasion none of the solutions removed detectable toxin. The reasons for this were not clear. Generally, the higher the concentration of ethanol, the better the desorption of toxin from charcoal.

Gel filtration. Chromatography with molecular sieving gels separated the toxin into multiple forms. Plots of toxic activity against fraction number yielded several peaks. This was demonstrated with both Sephadex G-15 and Bio-Gel P-2, but the P-2 gave better resolution of the toxins. Repeat chromatography of the collected toxic fractions gave very similar elution patterns, indicating the multiple peaks were not due to an inconsistent bioassay or an easily degraded molecule. The P-2 and G-15 gels which have exclusion limits of 1,800 and 1,500 daltons, respectively, never yielded toxin near their void volume. This suggests the toxic substances are below 1,500 daltons in size, although interactions leading to retardation of toxin molecules by the gel could give a low estimate of size.

Repeated partitioning of aqueous toxin solution with chloroform or ethyl acetate extracted varying amounts of the toxin, depending upon the age of the culture harvested. Toxin derived from 2-week-old cultures had approximately half of the total toxic activity extracted with 6 partitions of chloroform, whereas the same procedure gave several-fold less toxic activity from 7-week-old cultures. The temporal change in the amount of lipophyllic toxins recovered was related to certain peaks of toxic activity eluted from Bio-Gel P-2 columns. The first toxins to emerge from the column were partially soluble in chloroform, but toxins eluting later appeared to be completely insoluble. Gel filtration of chloroform extracts of toxin gave peaks of activity which corresponded with the earliest toxins to emerge from the column.

Toxins recovered from H. mammatum cultures were, therefore, related to the age at which the cultures were harvested. This was demonstrated clearly with Bio-Gel P-2 columns by comparing the elution patterns of

toxin harvested at 2, 4, and 7 weeks (Figure 1). Two week cultures yielded predominately the larger-sized toxins (largest substances are eluted first in gel filtration) that were partially soluble in chloroform. The toxins from 7 week cultures were less lipophyllic, smaller molecules, and toxins harvested after the intermediate 4 week period included those found at both 2 and 7 weeks.

Thin layer chromatography. Attempts at further purifying the toxin by TLC were disappointing. Generally, amounts of the toxin easily detected in assays overloaded the plate and produced poor resolution and streaking. This was most likely due to the high level of impurities found with the toxin even after passage through a Bio-Gel P-2 column.

The TLC solvent systems used by Schipper (41) gave results in my study similar to what he reported. The R_f values for toxic activity was the same as that reported by Schipper, but fewer areas of separated activity were detected. Most visible spots had R_f values similar to toxin. Ninhydrin positive or UV florescing materials were not correlated with areas of toxic activity.

Silicic acid column chromatography. A 1.5 x 90 cm glass column was slurry packed with 135 ml of Bio-Sil A (100 to 200 mesh, Bio-Rad Laboratories). A toxin sample from a Bio-Gel P-2 column was reduced to near dryness, dissolved in 1 to 2 ml of distilled water, and applied to the silicic acid column. Using a method modified from that of Schipper (41), the toxin was eluted from the column with n-hexane/isopropanol/ acetone (6:4:1), and 4.5 ml fractions were collected. After elution, most of the yellow pigmentation found in the toxin preparation appeared to remain in the column. A plot of toxic activity against fraction number resulted in several peaks, representing several host-specific toxins (Figure 2). The

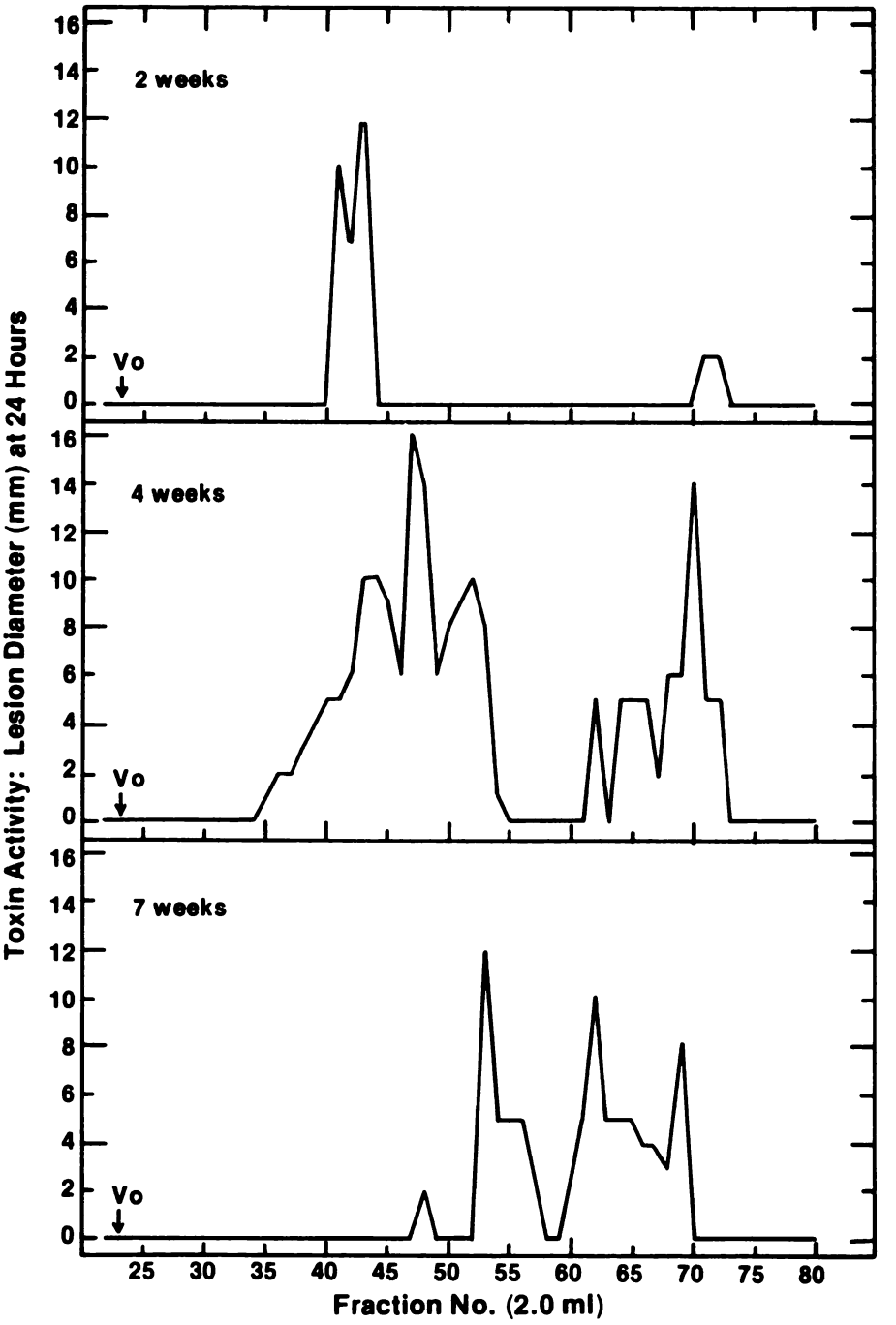

Figure 1. Effect of culture age on H. mammatum toxins recovered from culture fluids. Stationary cultures were grown on modified Fries medium at 24 C and harvested at 2, 4, and 7 weeks. The toxins were chromatographed with a Bio-Gel P-2 column (1.5 x 90 cm).

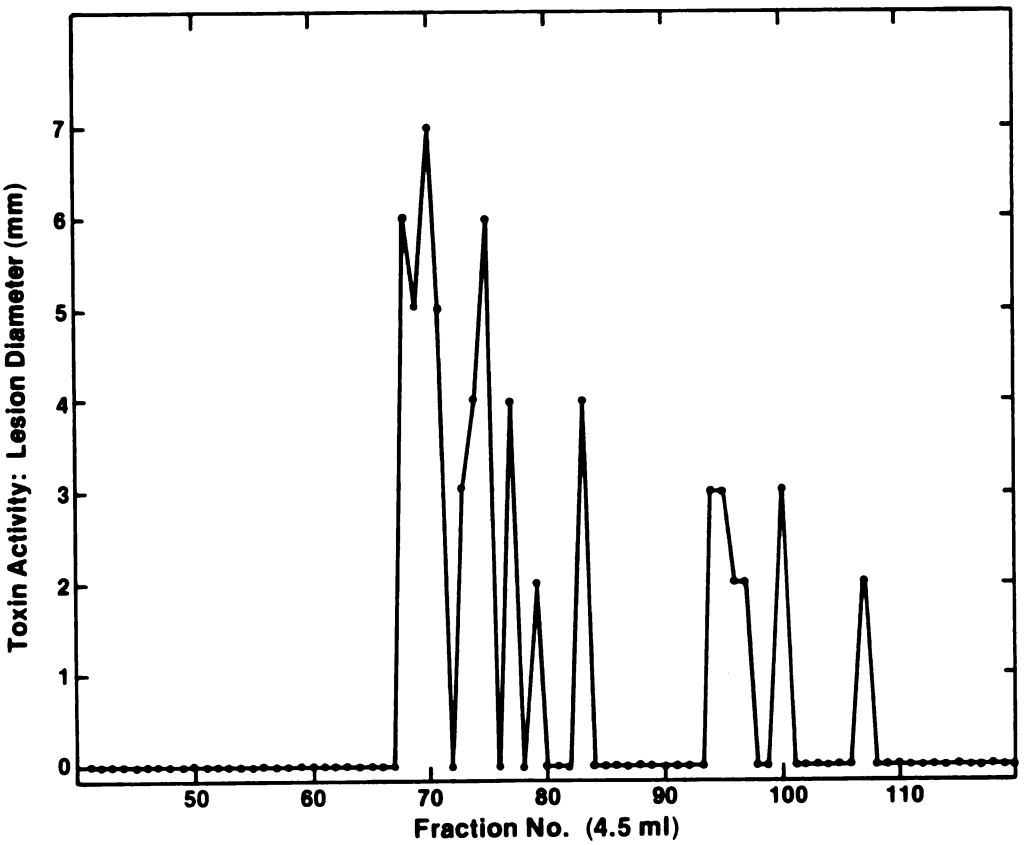

Figure 2. Chromatography of H. mammatum toxins on a silicic acid column. Stationary cultures were grown on modified Fries medium at 24 C and harvested at 7 weeks. The toxins were chromatographed on a 1.5 x 90 cm column packed with Bio-Sil A (100 to 200 mesh) and eluted with n-hexane/isopropanol/acetone (6:4:1).

findings were similar to those reported by Schipper who observed 7 host-specific toxins produced by H. mammatum.

High pressure liquid chromatography. The usefulness of HPLC to further purify the toxin was evaluated. One ml of toxin solution recovered from a P-2 column was reduced to 100 µl and injected into the HPLC column. The toxin was eluted with an ethanol gradient: 100% water for 1 min, followed by increasing ethanol concentration to 30% at 31 min, and finally a steeper gradient for 30 min more reaching 100% ethanol at 61 min. The machine shut down at 39.6 min because pressure to maintain the 1.0 ml per min flow rate exceeded 350 atm. The rest of the materials were eluted after re-programming for a 0.5 ml per min flow rate. Monitoring the eluate with UV at 280 nm indicated approximately 70 compounds eluted from the column, many exceeding full scale (1 AU) on the chart recorder. An assay of the fractions collected showed at least 5 host-specific toxins.

The toxin-containing fractions were collected, reduced to 100 µl, and rerun by HPLC. All conditions were the same as before, except this time a 1.0 ml per min flow rate was maintained for the entire elution. The pressure did, however, rise near 350 atm at 40 min. In both cases this transitory rise in pressure, due to increased flow resistance, occurred just prior to emergence of toxins from the column. Apparently, the viscosity of materials increased with rising concentrations of ethanol, and the pressure dropped when these materials eluted from the column.

The rechromatography resulted in a reduction in the number of UV absorbing substances detected to 6. And as shown by a marked reduction in degree of absorption, the amount of these 6 substances was substantially reduced as well. Moreover, the amount of toxin recovered was essentially

the same as that recovered from the first HPLC run. This demonstrated that HPLC achieved a large increase in the purity of the toxin.

Absorption spectra. A visible-UV spectrum from 800 to 200 nm was obtained for HPLC fractions recovered after the second passage through the machine. These were 7 sequential fractions, some which contained detectable toxin, and others which did not. A possible relationship was seen between toxic activity of the fractions and their absorption at 240 nm (Table 1). The lack of an exact correspondence between absorption and toxic activity could be due to variation in the bioassay. Other wavelengths of light did not show this correlation. The fractions absorbed no light in the visible range.

Electrophoresis. After the absorption spectra were run, the toxin-containing fractions were combined, reduced in volume and subjected to high voltage paper electrophoresis. An assay revealed that the toxin had migrated towards the positive electrode. All toxic acivity was found in a 3 cm section 5 to 8 cm from the origin. The amino acid standards were found in 3 areas: one spot 3.75 cm from the origin towards the cathode, 3 spots 31.5, 32.5, and 34.5 cm from the origin towards the cathode, and 2 spots 36.5 and 30.0 cm from the origin towards the anode. This experiment was repeated with a less purified toxin sample, giving very similar results.

Ion exchange. As might be expected from the negative charge seen in electrophoresis, the toxin did not bind to the cation exchangers (Dowex 50W-X2 and Sephadex SP C-25). The toxin was eluted at the void volume of the columns by distilled water. Pigmented impurities emerged from the column with the toxin.

Results from anion exchangers were not as simple. No toxic activity

Table 1. Light absorption and relative toxicity of fractions from HPLC of *H. mammatum* toxins[a]

	Optical Density		Toxin Activity
Fraction No.	240 nm	280 nm	(lesion dia., mm)
38	0.000	0.000	0
39	0.005	0.000	0
40	0.065	0.015	7
41	0.028	0.010	0
42	0.130	0.040	14
43	0.063	0.002	12

[a]Toxin was eluted from a Varian 4.1 x 300 mm MCH-10 column with a gradient of increasing ethanol concentration in water.

was recovered from the Dowex 2-X8 or the Sephadex QAE A-25. However, the yellow pigmentation could be eluted with 0.5 M NaCl. Because there was a possibility that the anion columns were degrading the toxin molecules, a weaker anion exchanger (Sephadex DEAE A-25) was tried. Elution with approximately 3 bed volumes (10 ml) of water left both toxin and yellow impurities on the column. The toxin was, however, readily eluted with 10 ml of 0.05 M NaCl, leaving the pigmentation at the top of the column. The colored materials could be eluted with 0.5 M NaCl. Purity of the toxin solution was visibly improved by the Sephadex DEAE exchanger.

Toxin Bioassays

Leaf puncture assay. The toxin solution recovered from a P-2 column caused necrosis on clone 5 leaves in dilutions out to 256^{-1}, while the undiluted toxin caused no necrosis on clone 2 leaves. Occasionally a highly concentrated toxin preparation did cause small necrotic lesions on the tolerant clone 2 leaves, but whether this was due to toxin or the concentrated impurities was not know. Repeated dilution end point assays indicated an approximate 250-fold difference in sensitivity to toxin between clone 2 and clone 5 leaves. The leaves from clone 3 plants were intermediate in sensitivity to toxin.

The effect of light or benzyl adenine (BA), a cytokinin, on the assay was studied. Different concentrations of BA were mixed with small volumes of toxin to give BA solutions of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 0 g per liter. Benzyl adenine had no effect on toxin-induced lesion development in leaf tissues. Leaves pretreated by permitting them to take up the various amounts of BA from vials and then assayed, also gave lesions not significantly different from controls. The influence of

light was investigated by taking 2 identically treated assays contained in humid chambers and placing one under a fluorescent light fixture and the other in a dark cabinet. There appeared to be a slight increase in lesion size with the light treatment, but the differences were not statistically significant.

A bioassay of 2 common substances gave surprising results. Both NaCl (1.0 M) and methanol gave a clone-selective reaction similar to that of the toxin (Table 2). The NaCl solution and methanol produced the largest necrotic lesions on the toxin-sensitive clone 5 leaves, and the smallest lesions on toxin-tolerant clone 2 leaves. The clone 3 leaves were of intermediate sensitivity, much like their reaction to toxin. However, the clonal differences were much less striking than were obtained with toxin.

Stem assay. H. mammatum attacks the trunk and branches of susceptible trees, not their leaves. In nature the toxin would presumably affect these woody parts during disease development. This made it desirable to study the effects of toxin on the stems of aspens. Also, it was important to know the relationship between leaf and stem sensitivity of a clone to toxin because the leaf assay has been used to screen for resistance to Hypoxylon canker (13,18). An untested assumption by these researchers was that the reaction of a leaf to toxin reflected the reaction of the stem.

The assay was performed by moistening a small ball of cotton with 50 µl of the test solution and placing it over a 2 mm wound that exposed the wood. The wound was made by removing a small plug of bark with a sawed-off hypodermic needle. The cotton ball was held in place by wrapping the stem with a strip of parafilm. Each treatment was replicated 4 times.

The stem of a tree gave a reaction similar to that of its leaves (Table 3). One week after the experiment began clone 5 stems treated with

Table 2. Toxicity of H. mammatum toxin, NaCl, and methanol to leaves from three clones of P. tremuloides

	Mean lesion diameter (mm)[a]					
	Clone 2		Clone 3		Clone 5	
Test Solution	24 h	48 h	24 h	48 h	24 h	48 h
1.0 M NaCl	b	b	b	2.75	b	3.25
Toxin	0	1.0	0	3.5	7.0	14.0
Methanol	3.25	3.5	5.0	7.0	7.5	9.0
Water	0	1.0	0	0.75	0	1.0

[a]Each value is the mean of 4 replicates from the leaf puncture assay. Lesions were measured 24 and 48 h after application of 10 µl of test solution.

[b]There was a 3 mm water-soaked area under the test sample, but there was no necrosis.

Table 3. Effect of *H. mammatum* toxin, methanol, and water on stems of three clones of *P. tremuloides*[a]

Test Solution	Responses		
	Clone 2	Clone 3	Clone 5
Water	Wound callus (5 mm dia.)	Wound callus (5 mm dia.)	Wound callus (6 mm dia.)
Methanol	Oval sunken lesions (4 to 6 mm long) surrounded by callus	Spindle-shaped sunken lesions (6 to 8 mm long) surrounded by callus	Oval or spindle-shaped sunken lesions (10 to 11 mm long) surrounded by callus
Toxin	Wound callus (5 mm dia.), no lesions	Black rings (1 to 3 mm) around the wound under the bark, no callus	Black oval sunken lesions (25 to 30 mm), no callus

[a]Stems were treated by moistening a small ball of cotton with 50 µl of test solution and placing it over a 2 mm wound that exposed the wood. The cotton was held in place by wrapping the stems with parafilm. Each treatment was replicated 4 times.

toxin had 25 to 30 mm black sunken lesions centered around the wound. The clone 3 stems treated with toxin had a 1 to 3 mm ring of necrotic sapwood around the wound. Neither clone 5 nor clone 3 developed any wound callus with toxin. The toxin-treated clone 2 stems reacted the same as water controls; callus was formed around the wound and there was no necrosis. Treatment with methanol produced sunken lesions of the same magnitude seen in leaf bioassays. All methanol-treated wounds also produced wound callus. At 2 weeks the water and methanol treatments, and the toxin-treated clone 2 stems were developing more callus and healing. At 2 weeks the toxin-treated stems of clones 5 and 3 appeared to be no different than at 1 week.

The severity of Hypoxylon canker has been directly correlated to moisture stress. A modest experiment was carried out to determine whether or not moisture stress caused increased sensitivity to toxin. Stems of four clone 2 and two clone 5 plants were treated with toxin, as described above, but this time the plants were watered only after obvious wilting occurred. Control plants received ample water. After 1 week the water-stressed clone 2 and clone 5 plants had lesions identical to their respective controls. Evidently, drought conditions had no effect on toxin sensitivity.

Dormant potted aspen plants held outside in a cold frame, were stem assayed. Stems were treated with toxin as described above. This was during early December when daily temperatures were around 7 C. Six young trees, 2 each of clone 2, clone 3, and clone 5 were tested with both water and toxin. No lesions developed on any plant and callus was absent. Toxin and water treatments were similar on all clones. These aspen plant had given a typical host-specific reaction to toxin the

previous summer. The dormant plants appeared to be insensitive to the toxin.

Leaf petiole assay. A more sensitive bioassay resulted when toxin test solution was applied to the cut petiole of an excised leaf. The 10 µl sample was placed on the petiole end and was taken up by the leaf. The treated leaves were then placed in vials contained within a humid chamber as before. Twenty-four hours later the toxin-treated clone 5 leaves exhibited degrees of necrosis from scattered flecks to complete necrosis of the leaf. Toxin-treated clone 2 leaves and the water controls showed no necrosis.

To determine the speed with which toxin would be distributed in a petiole-treated leaf, cuts were made perpendicular to the midvein 3, 1, 1/2, and 0 h after toxin application. All sections from leaves cut 3, 1, and 1/2 h after treatment were totally necrotic, indicating the toxin had spread throughout the leaf by these times. However, the leaf that was cut immediately after exposure to toxin also developed considerable necrosis. The actual time to complete the manipulation was 3 to 4 min, and during this time enough toxin had moved up the leaf prior to making the cut, to cause complete necrosis in the basal half and about 40% necrosis of the apical half by 20 h. Apparently, transpiration moves toxin very rapidly through aspen leaves.

Dry weights were determined for toxin present in selected fractions from a Bio-Gel P-2 column fractions, and the toxin was used in a dilution end point assay. Diluting the toxin in 10-fold increments, the highest dilution that showed some toxicity to clone 5 leaves was 10^{-2}. No necrosis was seen in clone 2 replicates. A leaf puncture assay performed at the same time could only detect the undiluted toxin. The dry weight

of the toxin preparation was 0.1 mg per ml, meaning the petiole assay detected toxin in a 1.0 µg per ml solution. In other words, a 10 µl impure sample of the toxin containing 10 ng of materials caused some necrosis in sensitive leaves.

When toxin-treated leaves of clone 5 were submerged in water, they did not develop the characteristic black necrosis. Instead, both the toxin-treated and control leaves had brown mottle. Submersion was accomplished by placing tubing on the leaf in a water-filled Petri dish. Inhibition of phenolic oxidation was assumed to be responsible (34), but the possible involvement of carbon dioxide, ethylene, or osmotic effects was not investigated.

In addition to being a more sensitive method, the leaf petiole bioassay appeared to be less affected by toxic impurities in the preparation. Ten µl samples of 0.5 M NaCl, 0.5 N HCl, and 0.5 N NaOH all failed to produce necrosis in either clone 5 or clone 2 leaves, but did cause considerable necrosis in the leaf puncture assay. The petiole assay detected host-specific toxin activity (necrosis only on toxin-treated clone 5, but not on treated clone 2 leaves) when 10 µl drops of solutions containing toxin plus 0.5 M NaCl, 0.5 N HCl, or 0.5 N NaOH. The assay results were the same as when only the toxin was present.

With the petiole assay, applied samples were distributed throughout the leaf, resulting in dilution of assay materials. This distribution was probably responsible for the lack of injury caused by contaminates in the petiole assay. Also, all the toxin can enter the leaf, unlike the puncture assay where most of the sample remains on the leaf surface. Amounts of toxin 1000 times that required to cause slight necrosis in clone 5 leaves did produce a similar degree of necrosis in clone 2 leaves.

Effects of Toxin on Tissues

Toxin-induced changes in respiration. Host-specific toxins produce many changes in susceptible tissues that are caused by fungal infections; examples are increases in respiration and in leakage of electrolytes (36). Treatments consisted of vacuum infiltration of leaf disks with distilled water or with toxin solution. Each treatment had two replicates for each clone. Measurements of rates of O_2 uptake with a Warburg apparatus showed that toxin-treated clone 5 tissues had 37% higher respiration rate than did control tissues (Figure 3). The toxin-treated clone 2 tissues and water control tissues gave similar results, yielding an O_2 consumption rate near 3 µl per min. The toxin-treated clone 5 leaf disks took up O_2 at aproximately 4 µl per min. The toxin-induced increase in respiration was evident at the first reading (60 min after infiltration). A repeat experiment gave similar results.

Toxin-induced changes in electrolyte leakage. Other toxins are known to cause leakage of electrolytes from susceptible tissues, and this has been used as a bioassay (37). Early attempts at detecting changes in electrolyte leakage caused by H. mammatum toxin were unsuccessful. When clone 2 and clone 5 leaf disks were infiltrated with distilled water or toxin solutions, as in the respiration experiments, no differences were seen between the treatments. Vials containing the leaf disks from the different treatments all gave conductivity measurements of 22 to 30 µmhos at 25 h. Leaf disks were then vacuum infiltrated and held in the toxin solutions for 1 h before they were rinsed and monitored for leakage; there was a slight increase by 24 h in electrolyte leakage of clone 5 disks treated with toxin solution. The disks also showed some necrosis.

Leaves from clone 2 and clone 5 plants were allowed to take up 10 µl

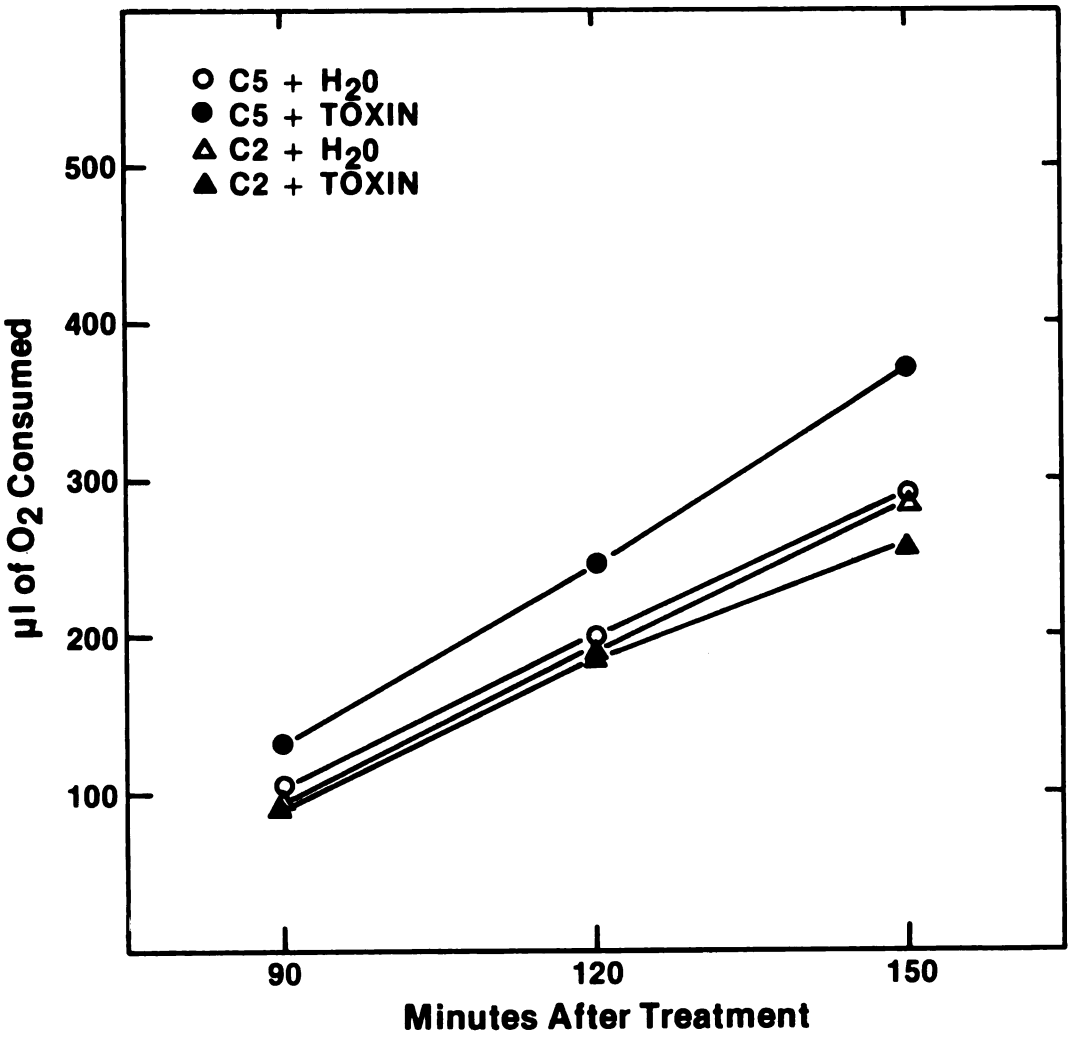

Figure 3. Effect of H. mammatum toxin on O_2 consumption by leaves of P. tremuloides clones 2 and 5 (C2 and C5). Leaf disks (1 cm in diameter) were infiltrated at reduced pressure with toxin solution or water. Leaf disks (0.1 g) were placed in Warburg flasks and the O_2 uptake measured manometrically. Each value is the mean of two replicates.

samples of either water or toxin solution through their cut petioles. Next the disks were cut from the leaves and were placed in vials with water. All treatments were replicated. The ambient solutions of toxin-treated clone 2 and water-treated clone 2 and clone 5 preparations increased in conductivity by approximately 0.9 μmhos per h. The ambient solutions of toxin-treated clone 5 leaves increased in conductivity by greater than 6.0 μmhos per h (Figure 4). Electrolyte leakage of the toxin-treated clone 5 leaf disks, however, increased at the same rate as the controls until 6 h after treatment, when there was an acceleration in leakage from toxin-treated tissue. By 24 h the vials containing the toxin-treated clone 5 leaf disks had an average conductivity of 127 μmhos per h, whereas the controls averaged around 30 μmhos per h. Some necrosis of toxin-treated clone 5 leaf disks was apparent by 5 h. At 24 h these leaf disks were necrotic in 40 to 100% of their area. There was no necrosis in the control and in the toxin-treated tolerant leaves.

Isolated *P. tremuloides* protoplasts. Isolated protoplasts are used frequently in studies of physiological phenomena in plants. A procedure for isolating aspen protoplasts was developed in my study so that the protoplasts could be used to study toxic effects. Protoplasts were isolated from aspen leaves by enzymatic digestion. The leaves used were approximately 1/4 the size of fully expanded leaves and had a shiny surface not seen in the more mature tissues. Leaves cut into 2 to 3 mm pieces were floated on enzyme solution in small Petri plates. The digestion solution contained 4% Cellulysin, 1% Macerase, and 1% (by volume) glusulase in 0.6 M sorbitol with 50 mM phosphate buffer, pH 7.0. The leaf pieces were vacuum infiltrated in the solution for 15 min, then incubated with the enzymes at 27 C for 24 h. After incubation, the

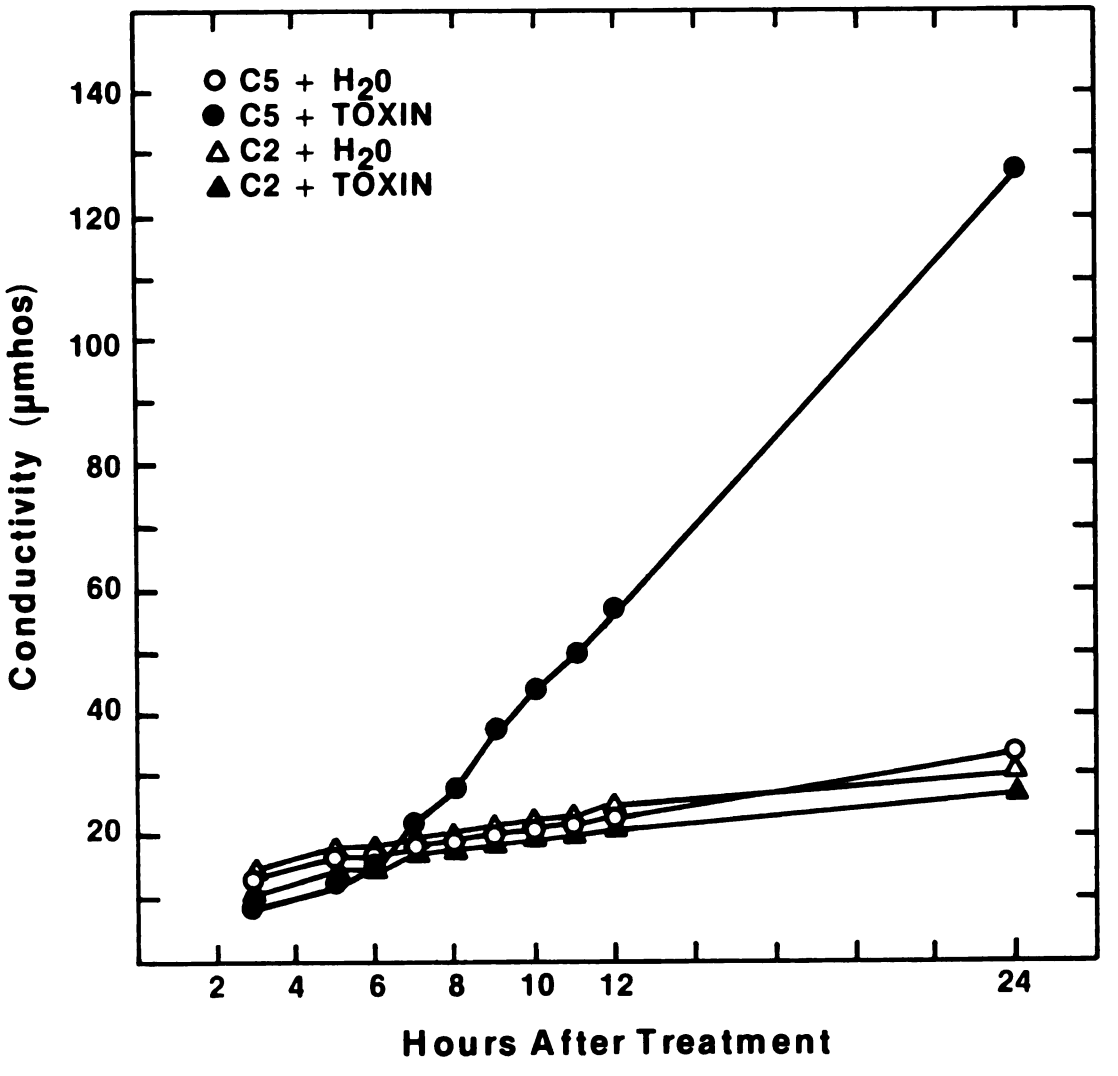

Figure 4. Effect of H. mammatum toxin on electrolyte leakage from leaf disks of P. tremuloides clones 2 and 5 (C2 and C5). Leaves were allowed to take up 10 µl of toxin solution through the cut petioles. Leaf disks (1 cm diameter) were then cut and 0.1 g of tissues were placed in vials containing 2.0 ml of water. Conductivity of ambient solutions was measured at intervals.

contents of the Petri plates were filtered though a layer of miracloth which retained plant debris but permitted the protoplasts to pass through. The filtrate was centrifuged at 50 xg for 4 min to pellet the protoplasts.

The protoplast pellet was resuspended in buffered 0.6 M sorbital containing toxin. At 24 h no differences were seen between toxin or water treatments in clone 2 or clone 5 protoplasts. Incubation of the toxin with the enzyme preparation did not alter the activity of the toxin. The protoplasts were apparently unaffected by toxin. There was a gradual death of cells over a 2 day period, as shown by staining with Evans blue. However, the dramatic necrosis and cell death so obvious in clone 5 leaf tissues was absent in isolated protoplasts.

Effects of Toxin on Aspen Hybrids

Leaf assay of aspen hybrids. The most convincing data showing that toxins have significance in disease development are based on genetic analyses of the host and pathogen (48). The following experiment with aspen hybrids was a preliminary attempt to determine the genetic control of sensitivity in aspen to the toxin. Hybrids of *Populus grandidentata* and *P. tremuloides* x *P. grandidentata* were tested for their reaction to *H. mammatum* toxin. I used a total of 36 plants from 6 different crosses made by Greg Reighard of the Forestry Department at Michigan State University. The plants were 4 months old and ranged from 30 to 80 cm tall when assayed. Leaves were tested by the leaf puncture and petiole assay methods with 10 µl samples of toxin. Some of the hybrids from crosses 559 x 699 and 157 x WENS were sensitive to the toxin; all the rest were insensitive (Table 4). One plant each from crosses 565 x 1-35 and 107 x 225B did give a slight reaction in the puncture assay at 48 h.

Table 4. Sensitivity of *Populus* hybrids to toxin from *H. mammatum*.

Cross	No. of progeny sensitive to toxin[a]	Total no. of progeny assayed
P. grandidentata x *P. tremuloides*		
(559 x 699)[b]	8	11
(107 x 225B)	0[c]	6
P. tremuloides x *P. grandidentata*		
(157 x WENS)	2	3
(565 x 1-35)	0[c]	9
P. grandidentata x *P. grandidentata*		
(107 x 307)	0	5
(559 x 307)	0	2

[a]Leaves were tested by the leaf puncture and petiole methods with 10 µl samples of toxin. The plants were 4 months old when assayed.

[b]These are reference numbers used to identify the clones.

[c]One plant did give a slight reaction in only the puncture assay at 48 h.

DISCUSSION

Isolation of *H. mammatum* toxins

Previous reports (5,18,41) were confirmed, showing that the *H. mammatum* toxin was soluble in polar solvents, heat stable, and composed of several similar host-specific toxins. Schipper (41) chromatographed culture filtrates from *H. mammatum* on silicic acid columns and recovered 7 different fractions that were toxic to leaves of *P. tremuloides*. I have obtained simiar results with a silicic acid column, indicating the presence of several different fractions, all with the same host-specific toxicity. Unlike work reported previously (18,41), the several different toxic molecules were also separated by gel filtration.

Some of the toxins were partially soluble in non-polar solvents, contrary to earlier reports (18,41). Repeated partitioning of aqueous toxin with ethyl acetate or chloroform extracted most of the toxins that were eluted first from Bio-Gel P-2 columns. The smaller forms of the toxin which emerged later from the column, appeared to be completely insoluble in non-polar solvents. Also, yields of the different toxins in liquid cultures varied with culture age. The larger toxins, partially-soluble in non-polar solvents, predominated in cultures at 2 weeks, but only small amounts were detected at 7 weeks. Differences in fungal isolates and culture media used by different workers could influence the nature of toxins produced by *H. mammatum*.

Several problems with thin layer chromatography of partially

purified toxin were encountered. Impurities in the toxin preparation made it easy to overload the plates and give poor resolution or streaking. When smaller amounts of the toxin-containing preparations were used, the toxic spots on the plates were difficult to detect in the bioassay. My results with the TLC solvent systems used by Schipper (41) gave R_f values for spots with toxic activity which were similar to his reported values, but I observed only 3 distinct areas with selective toxicity. Good separations could possibly spread the various toxins, resulting in a reduction of activity per area beyond the dilution end point of the assay. Contrary to the report of French (18), there was no consistent correlation between UV-fluorescence and selective toxicity. The toxin also appeared to be ninhydrin negative.

All forms of the toxin appeared to be acidic molecules. At physiological pH levels the toxins had a negative charge that caused movement in an electrical field and binding to anion exchangers. Neutral or basic forms of the toxin were not seen. Toxin migration in electrophoresis was less than that of negatively-charged aspartic and glutamic amino acids. This may have resulted from the friction of chromatographing a large molecule, or from the presence of only a partial net negative charge on the toxins. Strong anion exchangers apparently degraded the toxin molecules; even elution with 0.5 M NaCl did not recover toxic activity. The toxins bound to the weak exchanger Sephadex DEAE, and toxic activity was eluted with 0.05 M NaCl, but not with water.

Correlative evidence indicated the toxins absorb UV light at 240 nm. The absorption spectra of highly purified fractions from HPLC showed increases in absorption at 240 nm directly related to the toxic activity of the fractions. This absorption was observed in all the different

forms of the toxin separated by HPLC.

Adsoprtion and subsequent desoprtion of toxin with activated charcoal was useful in the early stages of purification, but the largest increase in purity was obtained with HPLC. A greater than 10-fold reduction in contaminates was achieved without a reduction in toxic activity. Chromatography of the toxin with Sephadex DEAE allowed elution of toxin from the column without removing the visible materials from the column. When the toxin recovered from gel filtration was subjected to ion exchange chromatography on DEAE and then chromatographed by HPLC through a C_{18} reverse phase column, the purity of the toxin preparation was substantially improved.

Toxin Bioassays

The leaf puncture assay method showed that leaves from P. tremuloides clone 2 tolerated at least 250-fold larger amounts of toxin than did leaves of clone 5. Treatment with light, darkness, or various concentrations of benzyl adenine did not appear to affect the results of the puncture assay; neither the severity of leaf necrosis nor the clonal specificity was changed. Impurities, however, did affect the bioassay through their toxic effects on leaves. Moreover, clones 2 and 5 differed in sensitivity to 1.0 M NaCl and methanol. The specificity of host-dependent lesion formation in leaves caused by the NaCl solution or methanol was similar to that for the toxin; however, the differences between clone 2 and 5 were not as great with NaCl and methanol as with toxin. The significance of this host-selective reaction to NaCl and methanol in the leaf puncture assay is not clear. A similar phenomenon was observed with the stem assay, but not with the petiole assay.

Stem assay results for clones 2, 3, and 5 showed that the toxin had the same selective effects on stems as on leaves. Establishment of this relationship is important because it is usually much easier to use leaves than stems to study toxic action and screen for resistance. Toxin inhibited the formation of wound callus in stems, as reported by other investigators (24,41). The lack of callus in toxin-treated wounded stems probably is not caused by simple injury of the bark. In contrast to the results of toxin-treatment, stem necrosis caused by methanol eventually callused and healed. Evidently, the toxin has a prolonged effect on wound callus formation in toxin-sensitive stems.

No lesions developed when toxin was applied to stems of dormant plants (clones 2 and 5). This correlates with resistance of dormant stems to infection by H. mammatum (7). The reason for this apparent insensitivity of dormant plants to toxin and to infection is not obvious. Temperatures during the toxin assay were above freezing. Stems of growing clone 5 plants in the greenhouse at the same time developed normal necrotic lesions after toxin was applied. Perhaps a physiological process necessary for susceptibility to the toxin is inactive in dormant tissues.

French (18) observed that plants of clone 2 and 5 were equally susceptible to H. mammatum when inoculated artificially. However, these clones differ greatly in their sensitivity to the toxin. Therefore, if the toxin is necessary for H. mammatum infection of aspen as hypothesized by Schipper (40), an effect of toxin other than necrosis must be important to the infection process. Results of toxin treatment of stems of drought-stressed plants is consistent with this. Although many workers have reported that susceptibility of aspen to H. mammatum infection

increases directly with moisture stress, the plants that were under considerable moisture stress did not increase in sensitivity to toxin. The necrotic effect of the toxin alone could not be responsible for the increased susceptibility to infection. Bagga and Smalley (5) reported that inoculations with ascospores of H. mammatum were not successful unless toxin was added with the inoculum. An interesting experiment would be to inoculate the stems of toxin-tolerant clone 2 aspens with ascospores and toxin, and see if a successful infection could develop without the initial necrotic lesion. Possibly, the role of the H. mammatum metabolite in the infection process is to induce compatibility in susceptible hosts, similar to that claimed by Oku et al. (31) for the non-toxic determinant of pathogenicity produced by Mycosphaerella pinodes.

The leaf petiole assay was more sensitive to toxin, yet was less affected by impurities than was the leaf puncture assay. Uptake of the entire sample into the leaf and dilution of the solutes throughout the lamina were probably why the petiole assay was more sensitive and selective. Application of a test solution to the petiole was, however, more time consuming and required more leaves than did the puncture assay, in which 4 samples can be tested on one leaf. The leaf puncture assay is preferred with partially purified toxin and for experiments requiring assays of many samples. The leaf petiole method should be used when assaying solutions with high levels of impurities or with very low levels of toxic activity. The petiole application method is more precise than Schipper's leaf assay method because the petiole method treats each leaf with a measured amount of toxin solution.

Effects of Toxin on Aspen

Physiological changes induced by H. mammatum toxin were similar to the changes that occur with many plant infections (36). The toxin caused increases in respiration, electrolyte leakage, and necrosis in sensitive tissues of P. tremuloides. The increases in respiration were detected relatively early; within 60 min the toxin-treated leaf disks of the susceptible clone 5 had a respiration rate higher than controls. Electrolyte leakage, however, was not detected until 5 h later. Necrosis was first observed in leaf disks just prior to detection of increased leakage, and the development of the necrosis was roughly proportional to increases in electrolytes found in ambient solutions. Apparently, the change in plasmalemma permeability of cells in toxin-treated clone 5 leaf disks is a secondary effect of the toxin, resulting from cell death. Earlier changes in permeability cannot be ruled out, because leaf characteristics which prevented good vacuum infiltration of leaf disks could possibly retard electrolyte leakage as well. The quick increase in O_2 consumption of sensitive leaf disks indicates this effect was related more closely to the primary site of action of the toxin.

Protoplasts were successfully isolated from young aspen leaves. The isolated protoplasts were to be used to study the action of the toxin, but protoplasts isolated from tissues of clone 5 were not visibly affected by the toxin. The cause of this apparent loss of sensitivity to toxin by protoplasts when isolated from tissues is not known. The plasmalemma surface left by enzymatic digestion was probably changed. The distribution and configuration of functional molecules left on the membrane surface are as likely to be an expression of the digestion procedure as they are to reflect any inherent properties of the

plasmalemma (22). Perhaps a toxin receptor was modified or removed from the membrane surface during the enzymatic digestion which could render the protoplasts insensitive to toxin.

There was much variation in toxin sensitivity of aspen hybrids, and also between members of a toxin-sensitive hybrid. Unfortunately, the toxin sensitivity of the parents was not known. The P. grandidentata plants probably were all highly tolerant (18,38), but the sensitivity of P. tremuloides parents could have varied (18). Hybrids between P. grandidentata clones were all highly tolerant to the toxin. Progeny from P. tremuloides x P. grandidentata crosses contained toxin sensitive plants in 2 crosses and no sensitive plants in the other 2 crosses. Because toxin-sensitive hybrids came from both male and female P. tremuloides parents, sensitivity to toxin does not appear to be cytoplasmically inherited. Genetic control of aspen reaction to the toxin is likely to be complex, similar to genetic control of resistance to Hypoxylon canker (27,45,46).

Evidence presented in my study and elsewhere support the hypothesis that the host specific metabolites from H. mammatum are required by the fungus for canker development in Populus species. Several lines of evidence indicate that the toxins are a significant factor in disease development. P. tremuloides, the only species highly sensitive to the toxins, is also the species most susceptible to H. mammatum infection; all other species tested are highly tolerant of the toxins and also very resistant or immune to infection (7,18,38). Inoculations of wounded quaking aspen stems with H. mammatum ascospores result in canker development only when combined with topical applications of H. mammatum toxins (5). Similarly, when exogenous toxin is applied to mycelial

inoculations of an avirulent isolate of H. mammatum, successful infection occurs; no infection occurs with the avirulent or the normally virulent isolates when the toxins are removed (40). Isolates of H. mammatum that produce the most toxin in culture are also the most virulent when artificially inoculated on quaking aspen stems (18). Results from my study show that dorman aspen stems are apparently insensitive to the toxins; because dormant stems are also resistant to infection by H. mammatum (7), this data is consistent with the hypothesis that the toxins are necessary for canker development. Also, the extreme biological activity of the H. mammatum host-specific toxins, with only nanogram amounts necessary to produce effects in sensitive tissues, argues against just a fortuitous correlation of the toxins with Hypoxylon canker.

There are, however, observations that raise some doubts about the role of the toxins. The tolerance of different P. tremuloides clones to the necrotic effect of the toxins is not correlated with their resistance to infection by H. mammatum (18). Also, there is the question of how the toxins act in natural infections; H. mammatum ascospores do not contain toxin (41), and no vector of mycelium has been identified.

I suggest that the primary effect of the toxins is to induce compatibility in susceptible hosts; the necrotic effect of the toxins is secondary. The action of the toxins is to permit canker development to occur (5,40). Perhaps this is by reducing the ability of a tree to recognize the pathogen, or by elicitation of a host response necessary for canker development. Examination of the toxic effects of the fungal substances could be misleading when used as a method to screen for resistance to Hypoxylon canker.

Genetic studies on the control of pathogenicity, virulence, and

toxin production in H. mammatum, and genetic studies of canker resistance and toxin sensitivity in Populus species, would greatly improve the understanding of the role of the toxins in disease development. Only a genetic analysis of canker resistance has been reported (46). Obviously more information is needed on genetic control of these factors. Purification of the H. mammatum toxins to homogeneity and their characterization also is needed for thorough investigations of their action and role in disease. The relationship between Hypoxylon canker and insects of aspen also deserves more study.

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