Isolation of Toxins of *Hypoxylon mammatum* and Demonstration of Some Toxin Effects on Selected Clones of *Populus tremuloides*

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This work was supported in part by McIntyre-Stennis funds and by National Science Foundation Grant PCM-8100711. Michigan Agricultural Experiment Station Journal Series Article 11185.

Accepted for publication 12 January 1984.

ABSTRACT

Stermer, B. A., Scheffer, R. P., and Hart, J. H. 1984. Isolation of toxins of *Hypoxylon mammatum* and demonstration of some toxin effects on selected clones of *Populus tremuloides*. Phytopathology 74:654-658.

Hypoxylon mammatum produces in culture several acidic metabolites that are very toxic to its trembling aspen (*Populus tremuloides*) host; nonhost species are tolerant. Toxins were partially purified by extraction with solvents, absorption on charcoal, gel filtration, chromatography on silicic acid and DEAE columns, and HPLC. The relative proportions of the toxic metabolites changed with the age of cultures. Clones of *P. tremuloides* differed in sensitivity; the most resistant clone tolerated 1,000-fold higher concentrations of toxins than did a sensitive clone. Sensitivity/tolerance of stem tissue was correlated with leaf response, as indicated by application to wounded surfaces. For leaf bioassays, drops (10 μl) were applied to a wound on the leaf or to the cut ends of petioles. The latter bioassay, which distributed toxins throughout the leaf blade, was more sensitive. The toxins caused increases in O_2 uptake by leaves, followed by necrosis and electrolyte leakage. The data support the hypothesis that toxins of *H. mammatum* are significant determinants in disease development.

Hypoxylon mammatum (Wahl.) Miller causes a serious disease of quaking aspen (*Populus tremuloides* Michx.). Many randomly-selected isolates of the fungus have been shown to produce toxic metabolites (4) which may be involved in canker development (11). These low-molecular-weight metabolites are very toxic to *P. tremuloides*, which is highly susceptible to the fungus, but are relatively harmless to other plant species (4,11). Sensitivity to the metabolites varies among naturally-occurring clones of *P. tremuloides*. Thus, the metabolites have something in common with known host-selective toxins which are required for selective pathogenicity to certain genotypes of the host (8). The toxic metabolites of *H. mammatum* may be crucial factors for infection, but this has not been established.

Both French (4) and Schipper (11) reported species-selective toxicity of the metabolites from *H. mammatum*. Aspen (*P. tremuloides*) was shown to be very sensitive, whereas *P. grandidentata* and *P. maximowiczii* gave a slight response, and three other *Populus* species were insensitive to concentrated culture filtrates (4). The leaves of seven other woody species were tested and were insensitive. The list of unaffected species of woody and herbaceous plants has since been extended (*unpublished*); no highly sensitive species other than *P. tremuloides* were found. Further, French found that *P. deltoides* and the insensitive clones of *P. tremuloides* tolerated a 1,000-fold higher concentration of a relatively crude toxin preparation than did a sensitive clone of *P. tremuloides*. However, the relationship between clonal sensitivity to toxins and susceptibility to the fungus is uncertain. There is another disease involving a known host-selective toxin that has a complex relationship with host clones which vary in sensitivity (9).

The purpose of this study was to develop reliable methods to purify the toxins, to examine some of their effects on aspen tissues, and to evaluate several toxic effects for possible use in improved assays. Further work is underway. The data to date support the hypothesis that toxins of *H. mammatum* have a role in canker development and host-selectivity. An abstract describing some of the work was published (13).

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MATERIALS AND METHODS

Young aspen trees were obtained from root cuttings of naturally occurring clones of *P. tremuloides* in Michigan. The clones were classified by leaf wound assays as highly sensitive (clone 5), highly tolerant (clone 2), or intermediate (clone 3) in reaction to toxins of *H. mammatum* (4). The root cuttings developed sprouts which were excised, rooted and grown in pots in the greenhouse. A photoperiod of at least 16 hr was maintained with supplemental illumination.

The isolate of *H. mammatum* (RL5A-7) used for toxin production originated from a single ascospore (4). One-liter Roux bottles containing 200 ml of a modified Fries medium (7) were inoculated with 1 ml of a suspension of fragmented mycelia. After 2–7 wk of stationary culture in low light at 24 C, the culture fluid was harvested by filtration through two layers of cheesecloth and a layer of Miracloth (Chicopee Mills, Cornella, GA 30531). Culture filtrate (5 L) was concentrated under reduced pressure at 30–40 C to 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 was concentrated to ~2.5% of the original volume. This preparation was partitioned three times against equal volumes of chloroform and then three 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. Five grams of activated charcoal (HCl-washed, from Sigma Chemical Co., St. Louis, MO 63178) was added for each liter of the original filtrate volume, and the slurry was stirred overnight at 4 C. The charcoal slurry was centrifuged, the supernatant was discarded, and the pellet was resuspended in water and recentrifuged. Toxins were desorbed from charcoal by three washings with water-saturated *n*-butanol. The *n*-butanol fractions were combined, reduced to near dryness, and resuspended in 1–2 ml of water. The concentrated toxin preparation was then chromatographed through a Bio-Gel P-2 or Sephadex G-15 column (1.5 × 90 cm) with water as the solvent, at a flow rate of 0.1 ml/min. The fractions from the column were assayed for toxicity and the toxic fractions were combined. These were the partially purified toxins used in many experiments. Further purification procedures are described below in the Results section.

Toxins were detected in solutions by using a leaf bioassay. Excised leaves were scratched with needle points, and a 10-μl drop of test solution was placed on the wound. Excised leaves were incubated in petri dishes on glass beads to hold them above water. Sizes of the toxin-induced lesions were measured at 24 or 48 hr after treatment. All assays were replicated on leaves of both the sensitive and the tolerant clone; in some cases, leaves of a resistant species (eg, *P. deltoides*) were used. The toxin activity of a solution was expressed as a titer; the highest dilution that still showed toxicity in the wounded leaf bioassay by 48 hr was used as the titer of the solution.

A slurry-packed, 1.5 × 90-cm column of Bio-Sil A (150–200 μm [100–200 mesh]) was used for silicic acid chromatography, with a method modified from that of Schipper (11). Toxins in 1–2 ml of water were placed on the column, and the chromatogram was developed with *n*-hexane/isopropanol/acetone (6:4:1). Flow rate was 1.0 ml per min. Fractions (4.5 ml each) from the column were reduced to dryness under reduced pressure and suspended in water for assay.

A Varian LC 5060 HPLC (high performance liquid chromatography) instrument with a Whatman Co:Pell ODS precolumn and a Varian 4.1 × 300 mm MCH-10 column were used. Aqueous toxin samples (100 μl) were injected into the column and were eluted with aqueous solutions containing increasing concentrations of ethanol. Eluate from the column was monitored with a UV detector at 280 nm. Ethanol was removed from the fractions, which were then brought to equal volumes and assayed for toxin.

A Savant high-voltage paper electrophoresis apparatus was used to analyze the toxins. The buffer was pyridine/acetic acid/water (25:1:225) at pH 6.5 (6). The paper (Whatman no. 4, 115 cm long) was used with kerosene electrophoretic coolant at 7,000 V and 100 mA for 1 hr. Toxins were detected by cutting the paper into sections, eluting the pieces with water, and assaying the water extract for host-selective toxicity. A mixture of acidic, neutral, and basic amino acids were run simultaneously with the toxins as standards; they were detected by dipping the paper in a ninhydrin solution and heating after the toxin-containing segments were removed.

Glass Pasteur pipets (14.5 cm long) were used for ion exchange chromatography. The narrow end was plugged with glass wool to support the exchanger bed, and a serum bottle cap with 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 sample of the toxins (0.2–0.5 ml) was applied to the top of the column and eluted with water and with NaCl solutions (0.05 and 0.5 M).

Oxygen consumption was measured by Warburg manometry, in replicated experiments. Leaf disks (1 cm diameter) were enclosed in cheesecloth bags, weighted down, and infiltrated for 15 min under reduced pressure in water or toxin solution. The treated disks were rinsed, randomized, and placed in Warburg flasks (0.1 g per flask) which contained 20% KOH in the center well. Preparations were equilibrated in the dark at 30 C, and O_2 uptake in the dark was determined manometrically for at least 2.5 hr (14).

Leaves used in electrolyte leakage experiments were allowed to take up 10 μl of treatment solution that was applied to the cut ends of petioles. The petioles were then placed in vials containing water, and leaves were permitted to transpire for 30 min. Disks 1 cm in diameter were cut from the leaves and rinsed in distilled water. Random samples (0.1 g each) were placed in vials with 2.0 ml of distilled water. Electrolyte leakage from leaf disks was determined by measuring the conductivity of the ambient solution with a conductivity meter equipped with a pipet-type electrode ($K = 1.0$) (9).

RESULTS

Toxin isolation. The toxins were very soluble in methanol, ethanol, *n*-butanol, acetone, and water. They were relatively insoluble in nonpolar solvents, but low amounts of toxic activity were extracted from culture filtrates and other aqueous solutions by ethyl acetate or chloroform partitioning. Toxicity was not destroyed by autoclaving for 20 min.

Chromatography of the butanol extracts with Sephadex G-15 or Bio-Gel P-2 gels separated several forms of toxin; P-2 gave better resolution (Fig. 1). Repeated chromatography of the pooled toxic fractions from the column gave very similar elution patterns, indicating that multiple peaks did not result from inconsistent bioassays or from easily degraded molecules. Bio-Gel P-2 was used with many preparations of toxin, from cultures 2–7 wk old; the results were comparable, except as indicated in a later section. The P-2 and G-15 gels have exclusion limits of 1,800 and 1,500 daltons, and never yielded toxin near their void volume. Thus, the toxic substances appear to be $<$ 1,500 daltons in size.

The toxic fractions from a P-2 column were combined, concentrated, and chromatographed with a silicic acid column with *n*-hexane/isopropanol/acetone (6:4:1) as the solvent. After elution of the toxins, most of the yellow impurities were left in the column. A plot of toxic activity against fraction number showed five or more peaks of toxic activity with selectivity (Fig. 2). Five or more peaks were evident in repeat experiments.

HPLC was used for further purification of the toxins. The pooled toxic fractions from the P-2 column were injected into the HPLC column and eluted with a gradient of increasing ethanol concentration in water. The eluate was monitored by UV absorption at 280 nm. Many peaks were detected, and were bioassayed for toxin; five had selective toxicity. Rechromatography of the toxin-containing fractions resulted in a reduction to six in the

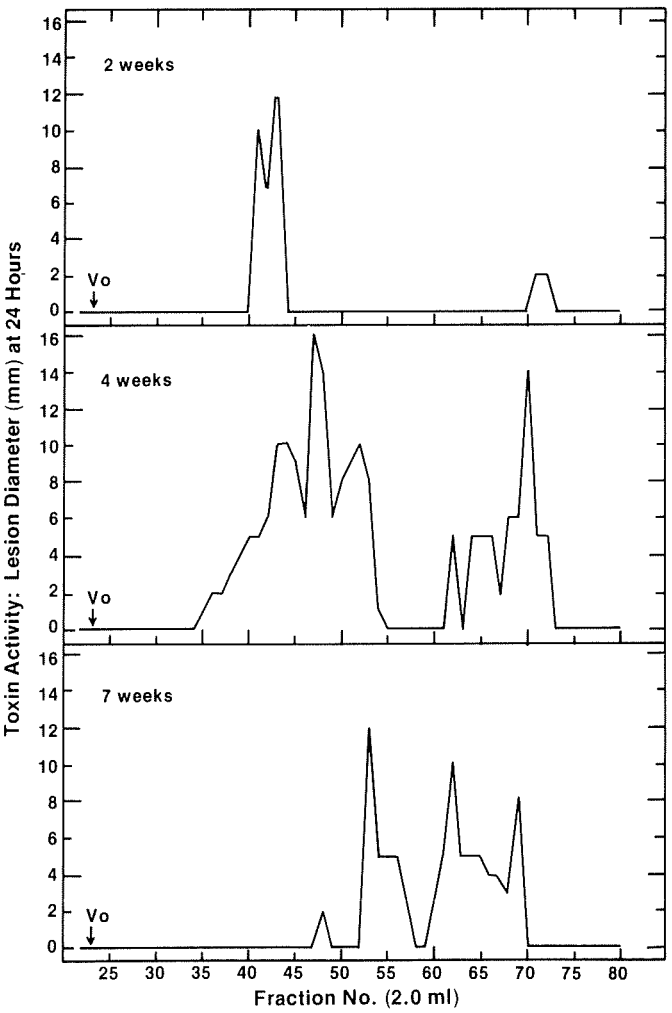

Fig. 1. Effect of culture age on toxins of *Hypoxylon mammatum* recovered from culture fluids. Stationary cultures were grown on modified Fries medium at 24 C and harvested at 2, 4, and 7 wk. The toxins were chromatographed on a Bio-Gel P-2 column (1.5 × 90-cm) and tested against wounded leaves of *Populus tremuloides* clones 5 (sensitive) and 2 (insensitive). Clone 2 showed no reaction. V_0 = void volume.

number of UV-absorbing substances detected, but only three fractions had selective toxicity in a bioassay. The total amount of toxin recovered was the same as that recovered from the first HPLC run, as determined by dilution bioassay of pooled fractions. The toxin-containing fractions were combined, reduced in volume, and subjected to high-voltage paper electrophoresis. The toxins migrated toward the positive electrode, as indicated by bioassays; the movement was slower than that of aspartic and glutamic acids and there was no separation of the several forms of toxin. This experiment was repeated with the toxin preparation from a Bio-Gel P-2 column; results were comparable.

Toxic fractions from the Bio-Gel P-2 column were subjected to ion-exchange chromatography. As expected, there was no binding of the toxin to cation exchangers. Also, no toxic activity was recovered, even after elution with 0.5 M NaCl, from the strong anion exchangers Sephadex QAE A-25 and Dowex 2-X8. However, all forms of the toxin were bound to the weak ion exchanger Sephadex DEAE A-25; toxin was not eluted with water but was eluted with three bed volumes of 0.05 M NaCl, leaving a brown pigment on the column.

Changes in toxins with age of cultures. The relative amounts of the several forms of toxin (Fig. 1) recovered from culture fluids of *H. mammatum* differed with the age of cultures at harvest time. This was demonstrated with Bio-Gel P-2 columns by comparing the elution patterns of toxin harvested at 2, 4, and 7 wk. The exact number of peaks with toxin activity is not clear in these data (Fig. 1), but shifts in toxic forms were evident. Two-week-old cultures yielded predominately the larger-sized toxin molecules, that were partially soluble in chloroform. The toxins from 7-wk cultures were less lipophyllic and had smaller molecules, and toxins harvested after the intermediate 4-wk period included those found at both 2 and 7 wk. Maximum toxin yield was at 4 wk as shown by other data (*unpublished*). This general pattern was confirmed by several experiments in which cultures of different ages were harvested.

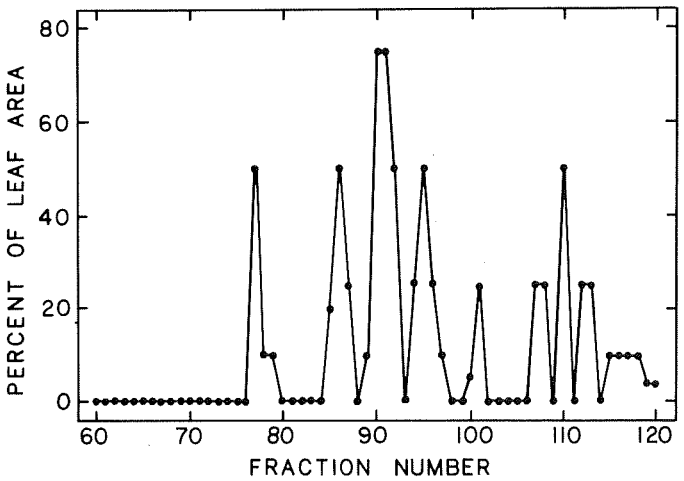

Fig. 2. Chromatography of toxins of *Hypoxylon mammatum* on a silicic acid column. Stationary cultures were grown on modified Fries medium at 24 C and harvested at 4 wk. The toxins were chromatographed on a 1.5 × 90-cm column packed with Bio-Sil A (100- to 200-mesh) and eluted with *n*-hexane/isopropanol/acetone (6:4:1). Fractions were assayed against wounded leaves of *Populus tremuloides* clone 5 (sensitive) and clone 2 (insensitive). The latter showed no reaction.

Effects of toxins on leaves and stems of *P. tremuloides*. Most of the work on toxin of *H. mammatum* has involved leaf reactions (3,4), although the fungus normally grows in the bark. Thus, it is important to compare the effects of the toxins on bark and leaves. Stems of clones 2, 3, and 5 were used; a wound which exposed the wood was made by removing a small plug of bark with the sharpened stub of a hypodermic needle. A small wad of cotton was moistened with 50 μl of the test solution, placed in the wound cavity, and held in place with a strip of Parafilm.

In the stem test (Table 1), the three clones showed the same relative sensitivities to toxins that they showed in leaf tests; clone 5 was most sensitive and clone 2 was most resistant. One week after exposure to toxins, stems of clone 5 had long, black, sunken lesions, 2.5–3.0 cm long, on each side of the wound; there was no callus. Toxin-treated stems of clone 3 had small necrotic zones around each wound, which had no callus. Toxin-treated stems of clone 2 reacted the same as did water controls; callus was formed around the wound and there was no necrosis. A control treatment with methanol produced sunken lesions on all three clones, but these lesions developed callus and were closing by 2 wk after treatment, as were the wounds on water-treated stems. In contrast, the toxin-treated stems of clones 3 and 5 had no healing or callus at 2 wk. Comparable results were obtained in a repeat experiment. Other experiments showed that moisture stress did not affect the sensitivity of clones 2 and 5 to toxins, and that stems of dormant plants (clones 2, 3, and 5) appeared to be insensitive to the toxins.

Sensitivity of leaf tissue was tested further by applying a 10-μl drop of toxin-containing solution to the cut petiole of an excised leaf. The drop was quickly taken up by the leaf, after which the leaf petiole was placed in a vial containing water and incubated in a humid chamber. After 24 hr, toxin-treated leaves of clone 5 showed extensive necrosis, often involving the whole leaf. Toxin-treated leaves of clone 2 and the water control leaves had no necrosis.

The petiole-uptake method was used to assay toxic activity in the eluate from a Bio-Gel P-2 column. Toxins were detected in a solution containing 1.0 μg dry weight per milliliter; a 10-μl sample of this toxin preparation, containing 10 ng of materials (including toxins), caused necrosis in sensitive (clone 5) leaves. No necrosis was seen in leaves of clone 2 which did not become necrotic even when 10.0 μg was applied to the leaf (a 1,000-fold increase). Concurrent assay by the leaf wounding method did not detect toxins in solutions with <0.1 mg dry weight per milliliter.

The petiole uptake and leaf wounding methods were used to compare necrosis induced by several common substances with necrosis induced by toxins. Solutions containing NaCl (0.5 M), HCl (0.5 N), or NaOH (0.5 N) caused no necrosis in leaves of clones 2 and 5, when applied as 10-μl drops to cut petioles; 10 μl of these solutions caused local, limited necrosis when applied to wounded leaf surfaces. Petiole application of 10-μl drops of each of these

TABLE 1. Effect of methanol and toxins of *Hypoxylon mammatum* on stems of three clones of *Populus tremuloides*[a]

	Respones of:		
Test solution	Clone 2	Clone 3	Clone 5
Water (control)	Wound callus (5 mm diameter)	Wound callus (5 mm diameter)	Wound callus (6 mm diameter)
Methanol	Oval sunken lesions (4–6 mm long) surrounded by callus	Spindle-shaped sunken lesions (6–8 mm long) surrounded by callus	Oval or spindle-shaped sunken lesions (10–11 mm long) surrounded by callus
Toxins in water[b]	Wound callus (5 mm diameter), no lesions	Black zones (1–3 mm) around the wound under the bark, no callus	Black oval sunken lesions (25–30 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 wound (2 mm in diameter) that exposed the wood. The cotton was held in place by wrapping the stems with Parafilm. Each treatment was replicated four times. Observations were taken 1 wk after treatment.
[b] Toxins were the pooled toxic fractions from a BioGel P-2 column, titer = 128.

solutions that also contained toxins caused extensive necrosis in leaves of clone 5, but none in leaves of clone 2. Succinic and oxalic acids at 0.1 M, applied to the wounded surface as 10-μl drops, caused small necrotic spots (<5 mm) on leaves of clones 2 and 5; lower concentrations (0.01 and 0.001 M) gave no necrosis.

Effects of toxins on electrolyte leakage and respiration. Effect of toxins on oxygen uptake was determined by the use of leaf disks, in replicated experiments. The leaf disks of clones 2 (tolerant) and 5 (sensitive) were infiltrated under reduced pressure with distilled water or with toxin solution from a Bio-Gel P-2 column (titer = 12.8). Toxin-treated tissues of clone 5 had higher respiration rates than did control tissues (Fig. 3). The toxin-treated tissues of clone 2 and water control tissues of clones 2 and 5 had similar uptake of O_2 (~30 μl per minute per gram of leaf tissue). Toxin-induced increases in respiration were evident 60 min after infiltration. The experiment was repeated with comparable results. Thus, toxin is capable of inducing this common response of plant tissue to infection; however, the response was not dramatic and was slow to develop. It may not be useful as the basis of a bioassay.

Effects of toxins on leakage of electrolytes from tissues were determined, as a possible basis for an improved assay. Leaves were allowed to take up 10 μl of water or toxin solution from a Bio-Gel P-2 column (titer = 256) through cut petioles. Disks (1 cm diameter) were then cut from the leaves and placed in vials with water. There were two vials for each treatment. There were no differences between water and toxin-treated tissues of clones 2 and 5 for the first 6 hr. Beginning at 6 hr after treatment, the ambient solutions of toxin-treated leaf disks of clone 5 increased in conductivity by >6.0 μmhos/hr (Fig. 4). By 24 hr the ambient solutions for the toxin-treated leaf disks of clone 5 had an average conductivity of 127 μmhos, whereas the toxin-treated leaf disks of clone 2 and the control solutions averaged ~30 μmhos. A little necrosis (<1.0% of the area) in toxin-treated leaf disks of clone 5 was apparent at 5 hr; at 24 hr, 40% or more of their area was necrotic. There was no necrosis in the controls or in the toxin-treated leaf disks of clone 2. The delay in response, which is similar to that for the host-selective toxin of *H. carbonum* (8), indicates that toxin-induced loss of electrolytes is questionable as the basis of a good bioassay.

DISCUSSION

H. mammatum produces several host-selective toxic molecules which can be separated and which may be similar to each other. Schipper (11) chromatographed culture filtrates from *H. mammatum* on silicic acid columns and recovered at least five, possibly seven, different fractions that were toxic to leaves of *P. tremuloides*; it is not clear that the fractions were assayed against both susceptible and resistant plants. We have obtained similar results with a silicic acid column, indicating several different toxins, all with selective toxicity to sensitive clones of *P. tremuloides*, but not to other plants. We also separated toxic materials by gel filtration, which previously has not been accomplished (4,11). Application of these toxic fractions to leaves showed that clone 2 of *P. tremuloides* tolerated at least 1,000-fold larger amounts of toxin than did clone 5.

Some of the toxins were partially soluble in nonpolar solvents, contrary to earlier reports (4,11). Repeated partitioning of aqueous toxin with ethyl acetate or chloroform extracted most of the toxins that came off in the earlier fractions from Bio-Gel P-2 columns. Smaller forms of the toxin, which emerged later from the column, appeared to be completely insoluble in nonpolar solvents. Also, yields of the different toxins in liquid cultures varied with culture age. The larger toxin molecules (partially-soluble in nonpolar solvents) predominated in cultures at 2 wk, but the smaller toxin molecules (insoluble in nonpolar solvents) predominated in the older cultures. Differences in fungal isolates and culture media used by different workers also could affect the type of toxins produced by *H. mammatum*.

H. mammatum causes lesions in stems rather than in leaves. However, most of the work on the toxins was with leaves (4,11,12), which are much easier experimental subjects. Our experiments showed that the stems of clones 2 (tolerant), 3 (intermediate), and 5 (sensitive) had the same relative sensitivities to toxins as did the leaves. Toxins caused necrosis and inhibited the formation of wound callus in stems of the sensitive clones, as reported for culture filtrates by other investigators (5,11). In our tests, toxins had no visible effects at the concentrations used on stems of the insensitive clone.

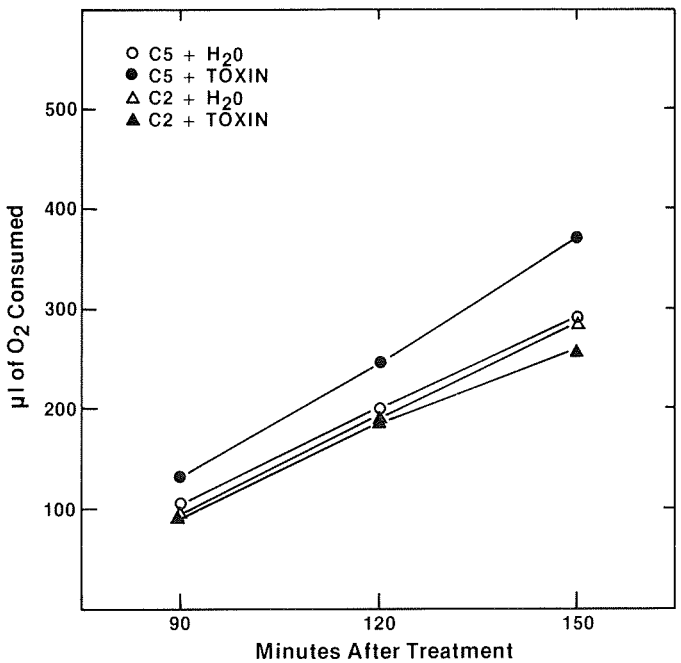

Fig. 3. Effect of toxins of *Hypoxylon mammatum* on O_2 consumption by leaves of clones 2 and 5 (C2 and C5) of *Populus tremuloides*. Leaf disks (1 cm in diameter) were infiltrated at reduced pressure with toxin solution (pooled toxic fractions from a Bio-Gel P-2 column, titer = 12.8) or water. Leaf disks (0.1 g) were placed in Warburg flasks and the O_2 uptake was measured manometrically. Each value is the mean of two replicates.

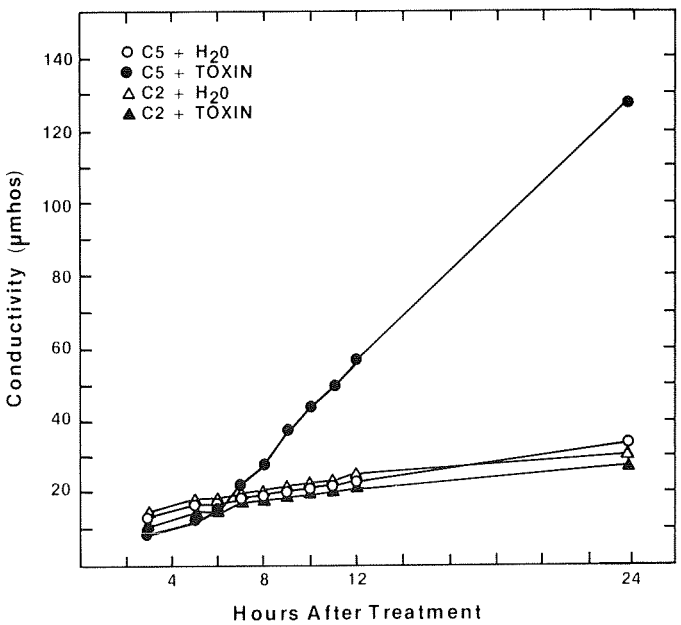

Fig. 4. Effect of toxins of *Hypoxylon mammatum* on electrolyte leakage from leaf disks of clones 2 and 5 (C2 and C5) of *Populus tremuloides*. Leaves were allowed to take up to 10 μl of toxin solution (pooled toxic fractions from a Bio-Gel P-2 column, titer = 256) through the cut petioles. Leaf disks (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. Each value is the mean for two flasks.

Good assays for toxins are very important for reliable results, and several methods were tested. Toxin-induced losses of electrolytes and increases in respiration appeared to be inadequate as the bases of assays. A convenient, but rather qualitative assay involves application of measured drops of a series of toxin dilutions to the scratched surfaces of leaves. A more precise assay was based on application of measured drops to the cut petiole of an excised leaf. The petiole method was more sensitive and reliable, and was less affected by the presence of other toxic substances. Uptake of the entire sample into the leaf and dilution of the solutes throughout the lamina probably account for greater sensitivity and selectivity of the petiole method. When drops of toxin are applied to the leaf blade, much of the toxin may be adsorbed to the leaf surface. Application of a test solution to the petiole was, however, more time consuming and required more leaves than did the leaf wound method, in which four samples can be tested on one leaf. An assay based on the leaf wound method is more practical for experiments requiring assays of many samples. The leaf petiole method can be used when precision is required, and for assays of solutions with very low levels of toxic activity.

There are several indications that the species-specific metabolites from *H. mammatum* are significant factors in disease development. *P. tremuloides*, the only species that is highly sensitive to the toxins, is also the species most susceptible to infection by *H. mammatum*; all other species tested are highly tolerant of the toxins and also very resistant or immune to infection (4,11). Inoculations of wounded stems of *P. tremuloides* with *H. mammatum* result in canker development only when combined with topical applications of metabolites of *H. mammatum* (1,10). Isolates of *H. mammatum* that produce the most toxin in culture are, in general, the most virulent when stems of *P. tremuloides* are inoculated (4). Crude culture extracts and isolated toxins cause bark collapse comparable to that occurring at sites of natural infection (11), and differ from that induced by toxic agents such as methanol, NaCl, or organic acids. Dormant aspen stems apparently are insensitive to the toxins and also are resistant to infection by *H. mammatum* (2); again, this is consistent with the hypothesis that the toxins are necessary for canker development. Finally, the very high biological activity of the toxins of *H. mammatum* argues against a fortuitous correlation of the toxins with disease development.

There still are questions about the role of the toxins of *H. mammatum* in disease development and the possible use of toxins in screening for disease resistance, as has been suggested (3,4,12). Clones of *P. tremuloides* differ in tolerance to the toxins, but this may not be correlated with their resistance to infection by the fungus (4). For example, clones 2 and 5 differed strikingly in toxin sensitivity, yet both developed cankers of similar size when inoculated with the isolate of *H. mammatum* used to produce toxin (4). However, inoculations were not successful in either clone unless fungal metabolites were added along with spores or mycelium (1,10). The toxins of *H. mammatum* clearly are species-specific; further study will be needed to understand the complex clonal effect. A similar complex situation also exists with certain clones of sugarcane, which may be either more or less susceptible to *H. sacchari* than is predicted by the reaction to its host-selective toxin (9).

Complete characterization of the toxins of *H. mammatum* is needed as a basis for further work, and is underway. Genetic studies on pathogenicity, virulence, and toxin production by *H. mammatum* will be needed to establish the role of toxin in disease development.

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