A Hypoxylon mammatum Pathotoxin Responsible for Canker Formation in Quaking Aspen

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ABSTRACT

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A toxic substance produced by the fungus *Hypoxylon mammatum* was responsible for canker formation in quaking aspen (*Populus tremuloides*). Cell-free extracts of culture medium and cankered host tissue caused inhibition of wound callus formation, bark necrosis and collapse, and distal vein necrosis in aspen leaves. Twenty-three of 24 *H. mammatum* isolates and two other fungus species, *Cenangium singulare* and *H. rubigosum*, produced substances in culture that were toxic to aspen leaves. Twenty-seven plant species were tested by leaf assay for toxin sensitivity. Only *P. tremuloides* was highly sensitive to the *H. mammatum* toxin. Several other poplars, one willow species, and bur oak also gave weak positive reactions. The toxic substance has a molecular weight of between 700 and 1,100 daltons, is heat stable, is soluble in polar solvents, and can be partially purified either by gel filtration, silica gel column and thin-layer chromatography, or high-vaccuum sublimation.

Hypoxylon mammatum (Wahl.) Mill. causes a canker on quaking aspen (*Populus tremuloides* Michx.) that is characterized by a flattened, sunken surface with a noncallused yellow-orange margin. How *H. mammatum* induces canker formation is not clear. The fungus grows in the sapwood of infected trees and invades the bark from within (9, 11), but because aspen bark contains fungitoxic compounds (10), the fungus apparently only invades dead bark. Therefore, a toxin that kills aspen bark in advance of the fungus might be the agent that causes the bark necrosis symptom of the canker.

During field and greenhouse experiments, the first symptoms of *H. mammatum* infection observed on aspen were an inhibition of wound callus followed by collapse and subsequent necrosis of bark around inoculated wounds before the mycelium had advanced beyond the wounded area (12). Hubbes (9) found that dialyzable substances from *H. mammatum* agar cultures inhibited callus formation in wounds on aspen bark. Bagga and Smalley (4) found that culture filtrates and killed *H. mammatum* agar cultures could cause blackening of wounded or nonwounded aspen bark. Therefore, the possibility that *H. mammatum* produces a toxin that causes bark collapse and bark necrosis in aspen was investigated.

MATERIALS AND METHODS

Aspen bioassay.—Initially, an aqueous extract of *H. mammatum* rye grain culture medium was prepared by soaking the grain in two parts (w/v) distilled water for 16 hr and then filtering it through a Millipore filter (0.22 μm pore size) (Millipore Corp., Bedford, MA 01730) to sterilize the extract. In subsequent experiments, the rye grain culture medium was homogenized in acetone with a Waring Blendor (Waring Prod. Div., Dynamics Corporation of America, New Hartford, CT 06057) for 5 min, the brei was stirred for 1 hr, and then filtered through Celite 545 (Johns-Manville, P.O. Box 5108, Denver, CO 80217). The acetone was removed by vacuum evaporation and the residue was dissolved in distilled water; insoluble materials were removed by filtration.

Stem wound bioassay.—Twenty greenhouse-grown aspen seedlings (104 ± 2 cm tall) (grown from seed collected from trees in Minnesota with *H. mammatum* cankers) were placed in a growth chamber [16 hr day (12,900 lux) at 24 C, 8 hr night at 18 C]. After removing all leaves from the basal 60-cm of each stem, rubber septum vial stoppers were placed around each stem at 15, 30, 45, and 60 cm above the soil and made watertight with RTV-11 silicone rubber (General Electric Company, Silicone Products Div., Waterford, NY 12188) to form reservoirs that held 1.5 ml (8). Two randomly chosen reservoirs on each stem were filled with the test extract and the other two reservoirs were filled with the control extract from sterile rye grain. After the reservoirs were filled, a 0.5-cm slit was made with a scalpel on the aspen stem below the surface of the extract in the reservoir. The extract in each reservoir was replenished as needed to keep the wounds submerged. The reservoirs were removed and the wounds examined after 4 days and again 3 and 7 days later. The experiment was repeated four times. Five additional trees were treated with the same extracts, but without wounding.

Aspen leaf bioassay.—Fully-expanded young aspen leaves were placed in 27-ml vials that contained 2 ml of

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toxin or control extract (enough to immerse the cut end of the petiole). Sixteen, 24, 48, 72, and 96 hr later the leaves were examined for vein necrosis. Leaves of various poplar and other plant species also were treated as described in the aspen leaf bioassay.

Impure freeze-dried extract that contained toxin was added to aspen leaf assay vials in concentrations ranging from 40 mg to 4×10^{-6} mg/ml distilled water. Aspen leaves placed in these solutions were examined as before.

Cut stem assay.—Aspen stem segments, 5 cm long (0.3 to 0.5 cm diameter), were placed in 27-ml vials that contained 2 ml of the toxin or control extract. The segments were examined daily for 7 days for signs of bark necrosis. In addition, some segments were removed from toxin and control extracts daily and allowed to dry to determine the effects of desiccation on toxin activity.

Sensitivity to toxin.—In addition to aspen, leaves from the following species were used to determine their susceptibility to the toxin substance: *Acer negundo* L. (box elder), *Berberis vulgaris* L. (European barberry), *Chenopodium album* L. (goosefoot), *Cirsium arvense* (L.) Scop. (Canada thistle), *Glycine max* (L.) Merr. (soybean), *Juglans nigra* L. (black walnut), *Lycopersicon esculentum* Mill. (tomato), *Malus sylvestris* Mill. (apple), *Phaseolus vulgaris* L. (kidney bean), *Picea glauca* (Moench) Voss (white spruce), *Poa pratensis* L. (Kentucky blue grass), *Populus alba* var. *pyramidalis* Bunge (Bolleana poplar), P. *balsamifera* Muench. (balsam poplar), *P. deltoides* Marsh. (cottonwood), *P. grandidentata* Michx. (big-toothed aspen), *Prunus virginiana* L. (choke cherry), *Quercus macrocarpa* Michx. (bur oak), *Rhus grabra* L. (sumac), *Ribes sativum* Syne (currant), *Salix* spp. (willow), *Sambucus canadensis* L. (common elder), *Syringa vulgaris* L. (common lilac), *Taxus canadensis* Marsh. (Canada yew), *Tilia americana* L. (basswood), and *Ulmus americana* L. (American elm).

Toxin production by Hypoxylon mammatum and other fungal genera.—Twenty-four isolates of *H. mammatum,* obtained from ascospores and from cankers collected throughout the Lake States and North Dakota, were used (Table 1) (1). Fourteen other fungi also were

TABLE 1. Toxin production by *Hypoxylon mammatum* isolates from the north-central region of the USA

Location[a]	*H. mammatum* isolate no.	Aspen leaf bioassay reaction[b] 24 hr	48 hr
North Dakota	1-1	+0.5	+1.4
	1-2	–	+0.3
	1-3	–	+0.2
Minnesota	2-4	+2.0	+3.0
	2-5	+0.1	+0.8
	2-6	+1.1	+2.1
	3-7	+0.3	+1.9
	3-8	+0.1	+1.0
	3-9	+0.7	+2.8
	4-10	+0.7	+1.5
	4-11	+0.1	+1.3
Wisconsin	5-12	+0.1	+0.6
	5-13	–	–
	5-14	+0.1	+1.0
	6-15	–	+0.8
	6-16	+0.1	+1.6
Michigan	7-17	+0.3	+1.1
	7-18	+0.6	+2.3
	7-19	+0.5	+1.5
	8-20	+0.5	+2.0
	8-21	+0.5	+1.0
	8-22	–	+0.4
NC-68	23	+0.1	+1.0
Conidial	24	–	+0.1

[a]Locations and isolate numbers refer to those used in Anderson and Schipper. 1975. Eur. J. For. Pathol. 5:216-224.

[b]Aspen leaf bioassay for *H. mammatum* toxin: minus (–) indicates no toxin reaction; plus (+) refers to a positive toxin reaction. The associated number refers to amount of leaf blade necrosis where 1 = 25%, 2 = 50%, 3 = 75%, and 4 = 100%.

TABLE 2. Toxin production by various fungi that cause tree cankers

Fungus	Isolated from	Aspen leaf assay
Hypoxylon mammatum (Wahl.) Mill.	Ascospores (Minnesota)	+3.7[a]
Cenangium singulare (Rehm.) Davids.	Aspen canker (Colorado)	+3.3
Ceratocystis crassivaginata Griffin	Aspen canker (Minnesota)	+1.0
C. fimbriata Ell. & Halst.	Aspen canker (Minnesota)	–
C. pilifera (Fr.) C. Mor.	Aspen canker (Colorado)	+0.4
C. populina Hinds & Davids.	Aspen canker (Colorado)	+0.2
Diaporthe allegheniensis R. H. Arn.	Yellow birch canker (Michigan)	–
Endothia parasitica (Murr.) And. & And.	American chestnut canker	+0.5
Hypoxylon rubigosum Pers. ex Fr.	Sugar maple canker (Michigan)	+1.2
Nectria gallagania Bres.	Aspen canker (Minnesota)	+0.7
Schizophyllum commune Fr.	Sporophore (Minnesota)	–
Ceratocystis tremulo-aurea	Aspen canker (Minnesota)	+0.7
C. tremulo-aurea	Aspen canker (Colorado)	+0.5
Valsa ambiens	Yellow birch canker (Michigan)	–

[a]Aspen leaf bioassay for toxin production by tree-cankering pathogenic fungi: (a positive leaf assay means distal vein necrosis was present) 1 = up to 25% of leaf blade necrotic; 2 = up to 50% of leaf blade necrotic; 3 = up to 75% of leaf blade necrotic; and 4 = up to 100% of leaf blade necrotic (a negative assay means that no distal vein necrosis was present).

tested (Table 2). Extracts of these fungi were prepared by acetone extraction of cultures grown on rye grain.

Isolation of toxin from cankers.—Extracts from healthy and *Hypoxylon*-cankered aspen trees and from *H. mammatum* perithecial stroma were prepared by acetone extraction of air-dried bark, wood, and stroma, that had been ground to pass a 0.97-mm (20-mesh) screen in a Wiley mill (A. H. Thomas Company, P.O. Box 779, Philadelphia, PA 19105), then extracted with acetone as described previously. After the acetone was removed by vacuum evaporation, the water-soluble components of the extracts were tested for the toxic substance by the aspen leaf bioassay.

Toxin production in culture.—*Hypoxylon mammatum* was grown in 250 ml of aspen powder in distilled water (1:100, w/v), glucose-peptone in water (1:1:100, w/w/v), malt extract in water (2:100, w/v), or potato extract in water (2:100, w/v) in diffuse light at 25 C. At 1-wk intervals mycelium was recovered by filtration. Each culture filtrate was treated with acetone to remove proteins, evaporated under vacuum to near dryness, diluted to 50 ml with water, and tested for toxic activity by the aspen leaf bioassay. The 5-fold concentration was used in order to detect the toxin soon after its production began. At later intervals the toxin could be detected in unconcentrated filtrates.

The mycelium was homogenized in acetone, then removed by filtration. The extract was freed of acetone by vacuum evaporation, dissolved in 5 ml of water, and tested for toxic activity by the aspen leaf bioassay.

Partial purification of toxin.—Several alternative methods were used to partially purify the toxic extract. Column chromatography of *H. mammatum* rye grain extracts was done with Sephadex G-10, G-15, and G-25 (Pharmacia Fine Chemicals, Div. Pharmacia Inc., Piscataway, NJ 08854) and Bio-gel P-2 (Bio-Rad Laboratories, Richmond, CA 94804), with distilled water or 0.1 M phosphate buffer (pH 7) as the eluant. Fifty to 100 3-ml fractions, depending on the gel filtration medium used, were collected and tested for toxic activity with the aspen leaf bioassay. Toxin in a minimum volume of acetone was applied to a silica gel column and eluted with 60% petroleum ether (b.p. 60-70 C): 40% isopropyl alcohol. One-hundred-forty 5-ml fractions were collected, evaporated under vacuum to dryness, redissolved in an equal volume of water, and tested by the aspen leaf bioassay for toxic activity.

Extract dissolved in acetone was separated by thin-layer chromatography (TLC) with a variety of solvents (Fig. 5). After development was complete, plates were examined under an ultraviolet lamp and fluorescent bands were marked. Then each fluorescent and nonfluorescent portion was scraped separately from each plate, eluted with acetone, dried in vacuo, and redissolved in 5 ml water. Each fraction obtained in this manner was checked for toxic activity by the aspen leaf bioassay.

Extract dissolved in water was applied to a mixed-bed ion exchange resin column of Amberlite IR-400 and IRA-120 (Rhom and Haas Company, Philadelphia, PA 19105), and eluted with 200 ml distilled water and then with 200 ml of 1 M $(NH_4)_2CO_3$. The water fraction was evaporated under vacuum to near dryness and then diluted to the original volume of applied toxin. The ammonium carbonate fraction was heated on a steam bath until the ammonium carbonate was removed, then evaporated under vacuum to near dryness and diluted to the original volume. Each fraction was tested for toxin activity by the aspen leaf bioassay.

Additonal purification of the toxin was attempted by high-vacuum sublimation. Toxin prepared by acetone extraction from rye grain culture was evaporated to near dryness, placed in a sublimation chamber, then air-dried. Pressures below 1×10^{-6} Torr were obtained and the sublimation chamber was partially immersed in an oil bath heated to 65, 110, 160, and 180 C, and held at each temperature for 0.5 hr. The sublimate was dissolved in acetone at the end of each heating period. Each eluate and the residue from the 180 C treatment was evaporated in vacuo to remove acetone, dissolved in water, and tested for toxic activity by the aspen leaf bioassay.

RESULTS

Aspen bioassay.—Bark collapse and bark necrosis (Fig. 1) were found on 76% of the wounds treated with the *H. mammatum* rye grain extract (Table 3), callus formed

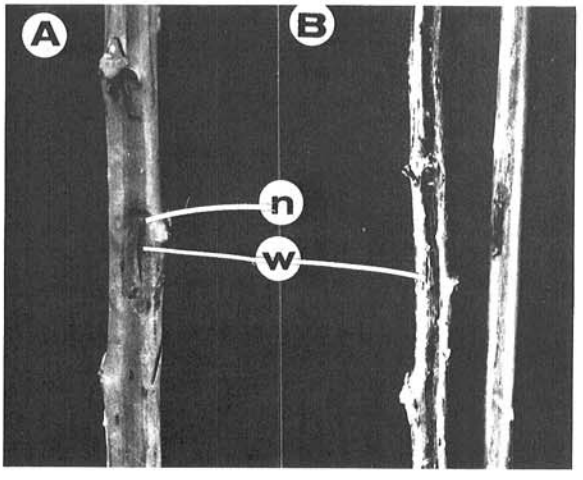

Fig. 1-(A, B). A) Aspen stem with a 0.5-cm-long wound treated with the *Hypoxylon mammatum* pathotoxin. Note the absence of callus in the wound and the discolored area of necrotic bark surrounding the wound. **B)** A 0.5-cm-long wound on a similar aspen stem treated with the control extract. Note the wound callus within the wound and the absence of discoloration and necrosis surrounding the wound (w = wound; n = necrotic bark).

TABLE 3. Effect of *Hypoxylon mammatum* rye grain culture extracts and control extracts on wounded aspen bark

Treatment[a]	Host response		
	Bark necrosis (%)	Wound callus (%)	No reaction (%)
H. mammatum extract	76	2	22
Sterile rye extract	0	93	7

[a]Treatment was applied in reservoirs to 80 trees for 4 days and observations were made after 7 additional days.

on 2% of the wounds, and no reaction was evident on 22% of the wounds. Wound callus developed on 93% of the wounds treated with extract of sterile rye grain. None of the control wounds developed bark collapse or bark necrosis. In control wounds, callus development was first visible 3 days after treatment as a yellow-white line in the wound, but in toxin-treated wounds the edge of the wound was black. Bark necrosis was evident around the wounds before the reservoirs were removed. Bark collapse was observed 3 days after reservoirs were removed and increased during the following week, but did not increase further. Bark collapse or necrosis did not develop on trees where reservoirs received toxin but no wound was made.

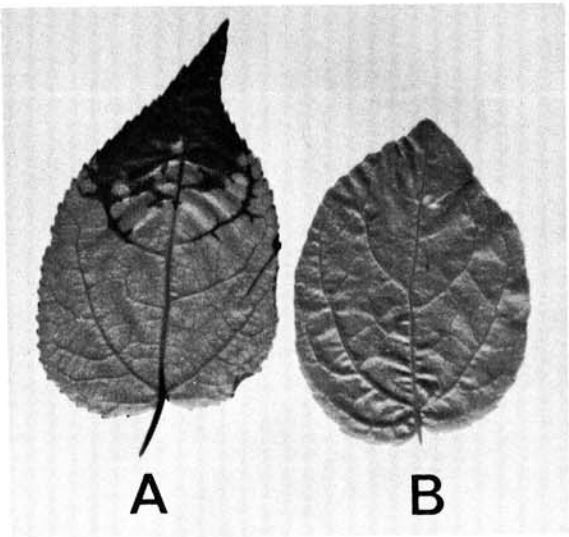

Fig. 2-(A, B). Aspen leaves treated in the aspen leaf bioassay for *Hypoxylon mammatum* toxin. Leaf **A)** was treated with a toxin-containing extract; leaf **B)** was treated with a control extract that did not contain any toxin. Note the distinctive necrosis on the toxin-treated leaf. The necrosis begins in veins as can be seen in some of the lower veins in the leaf, then spreads into the interveinal regions of the leaf blade.

When whole aspen leaves were placed in a 27-ml vial with a few milliliters of toxin-containing extract so that the petiole alone was immersed, vein necrosis at the distal end of the leaf appeared within 7 hr and spread over much of the leaf blade within 16 hr (Fig. 2). When the chemicals used to prepare the extracts as well as with extracts of sterile rye grain, were tested, either no leaf necrosis occurred or it occurred at the basal end of the leaf.

In the aspen stem segment bioassay, so long as the cuttings were left in the vials, only slight discoloration appeared at the base of the cuttings. However, within a few hours after the cuttings were removed from the vial and laid on filter paper, long, irregular areas of black necrosis and collapse appeared which extended from the base toward the apex of the cutting. Necrosis did not appear on cuttings exposed only to extracts from sterile rye grain or to distilled water.

Toxic activity was evident within 24 hr in the assays that contained from 40 mg to 2 mg/ml of impure extract. Leaves exposed to 4×10^{-3} mg per ml extract required 96 hr to develop necrosis.

Seven of the 27 plants tested (Table 4) were sensitive to the toxin, but only two of these were not species of *Populus*. In all cases, leaves of quaking aspen were more sensitive than any other plant species. Vein necrosis developed in 16 hr in quaking aspen leaves but developed more slowly in the other species.

Production of toxic substances by Hypoxylon mammatum and other genera.—Only one of the *H. mammatum* isolates did not produce detectable toxin as measured by the aspen leaf bioassay (Table 3). The 23 isolates that produced the toxic substance differed in the amount they produced: only five produced enough to make between 50 and 75% of the leaf blade necrotic. An isolate from a canker obtained near Baudette in northern Minnesota produced more toxin than any of the others. A conidial isolate that was nonpathogenic produced only barely detectable amounts of toxin. The one isolate that did not produce detectable toxin had been pathogenic in inoculation trials conducted earlier, but may have lost pathogenicity during culture.

In a separate experiment 14 isolates, including *H.*

TABLE 4. Susceptibility of various plant species to the *Hypoxylon mammatum* pathotoxin

Plant family/species	Common name	Leaf assay reaction	
		Toxin	Control
Salicaceae:			
Populus alba var. *pyramidalis*	Bolleana poplar	+1[a]	–[b]
P. balsamifera	Balsam poplar	+2	–
P. deltoides	Eastern cottonwood	+1	–
P. grandidentata	Bigtooth aspen	+2	–
P. tremuloides	Quaking aspen	+4	–
Salix sp. no. 1	Willow	+1	–
Salix sp. no. 2	Willow	–	–
Fagaceae:			
Quercus macrocarpa	Bur oak	+1	–

[a]A positive leaf assay means distal vein necrosis was present. 1 = up to 25% of leaf blade necrotic; 2 = up to 50% of leaf blade necrotic; 3 = up to 75% of leaf blade necrotic; and 4 = up to 100% of leaf blade necrotic.

[b]A negative assay means that no distal vein necrosis was present.

mammatum, were grown in rye grain culture and tested for toxin production by the aspen leaf bioassay method (Table 2). Only two of the isolates, *H. mammatum* and *Cenangium singulare*, produced large amounts of toxin. Chromatography (TLC) of both the *H. mammatum* toxin and the *C. singulare* toxin indicated that the two toxins migrated in an almost identical manner on TLC plates. *Cenangium singulare* is an important canker-causing pathogen of quaking aspen in Colorado. *Ceratocystis crassivaginata* and *H. rubigosum* both produced material in culture that gave positive reactions in the aspen leaf bioassay. *Ceratocystis pilifera, C. populina, C. tremmulo-aurea, Endothia parasitica,* and *Nectria gallagania* also produced detectable amounts of toxic material but the leaf assay reactions required 72 to 96 hr and were not identical with the reaction for the toxic substances from *H. mammatum* and *Cenangium singulare*.

Isolation of toxic substance from cankers.—Toxic materials were obtained from extracts prepared from perithecial stroma, from bark and wood within cankers, and from bark and wood in the 5 cm zone adjacent to cankers. Toxic activity was not detected in tissue obtained further away from the cankers or from tissue obtained from healthy trees. Ascospore extracts did not yield the toxic substance.

Toxic substance production in culture.—Small amounts of toxic activity could be detected in the culture filtrate after the first week of *H. mammatum* growth, and the titer of toxic activity increased in culture filtrates obtained after longer times. No toxic activity could be detected in extracts prepared from mycelium removed from the liquid medium.

In a separate experiment, liquid cultures consisting either of aspen wood powder suspension, glucose-peptone, or potato extract were compared with malt extract as media for toxic substance production. Extracts from the aspen powder suspension contained the most toxic substance, glucose-peptone and malt extract were intermediate, and potato extract was a poor medium for toxic substance production.

Isolation and partial purification of the toxic substances.—When extracts from rye grain culture of *H. mammatum* were eluted through Sephadex G-10, G-15, G-25, or Bio-gel P-2, toxic activity was detected as a major peak with a shoulder (Fig. 3). Results from both buffered and nonbuffered runs were identical, but buffer caused basal damage to leaves. The elution pattern of the toxin on Bio-gel P-2 was compared with that of raffinose, sucrose, and glucose. The toxin eluted between the void volume and raffinose, indicating that the molecular weight of the toxin was between 594 and 1,800 daltons, the exclusion limit of Bio-gel P-2 listed by the Bio-Rad Laboratories. Toxic fractions of *H. mammatum* toxin eluted through Bio-gel P-2 were pooled, dried, and analyzed by direct probe on a LKB mass spectrometer (Type 9000, LKB Produkter, Bromma 1, Sweden). Although the toxin preparations contained other compounds in addition to the toxic substance, and the mass of the highest-molecular-weight compound varied between preparations, none of the components found in the toxin preparations had a molecular ion with a mass higher than 1,100.

When toxin was separated on silica gel columns and petroleum ether-isopropyl alcohol (6/4, v/v) was the eluant, multiple peaks of toxic activity were detected by the aspen leaf bioassay (Fig. 4). These fractions of toxin activity appeared to correspond with toxin activity detected by TLC (Fig. 5). With TLC, however, great care had to be taken to remove all silica gel from the extracts because silica gel caused necrotic flecks in aspen leaves. The toxic activity sublimed only between 65 and 110 C. Chromatography (TLC) of the toxic sublimate showed that it contained a number of components, some of which were not toxic.

When toxin was eluted through a column of Amberlite IR-400 or IRA-120, all of the toxic activity was recovered in the water eluate. No toxin activity was detected in the ammonium carbonate eluate of resin.

DISCUSSION

Although several bioassay systems were tried, only the aspen stem wound bioassay, the aspen stem segment bioassay, and the aspen leaf bioassay were useful to detect the *H. mammatum* toxic substance. The aspen leaf bioassay was the simplest of the three; it required the fewest plants, and easily could be used to test a large number of samples for toxin. The toxic action in both the

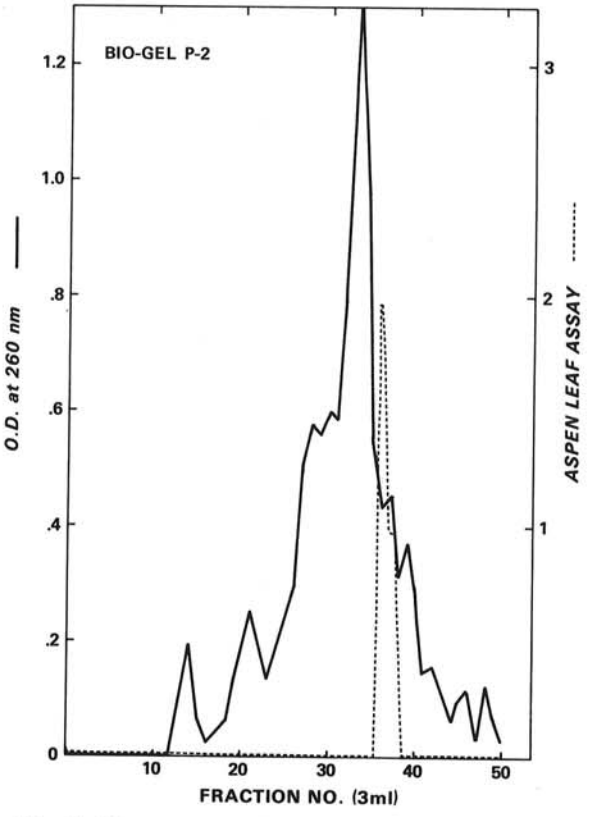

Fig. 3. Chromatogram (Bio-gel P-2) of the *Hypoxylon mammatum* toxin. Essentially all of the activity was contained in a single peak that eluted after most of the 260-nm-adsorbing material in the extract.

aspen leaves and in aspen stem cuttings was highly sensitive to moisture regime of the tissue. Thus, toxin was taken up by the leaf but the necrotic response to the toxin was delayed until leaf transpiration caused the leaf to be under moisture stress. In addition, bark necrosis on the stem segments treated with toxin did not appear until after the segment had been allowed to partially dry. Bier (6) and Bagga and Smalley (3) reported that *H. mammatum* cankers develop most rapidly when moisture is low in the bark of inoculated plants.

The aspen stem segment bioassay showed that the leaf vein necrosis probably was caused by the same toxic substances that caused stem bark necrosis and collapse. When left in sterile solutions for extended time periods, aspen stem segments developed basal callus but such callus did not develop on cuttings placed in solutions that were toxic in aspen leaf bioassays. Thus the toxic substances that cause vein necrosis and bark necrosis were probably the same as those that inhibited wound callus in intact trees inoculated with *H. mammatum*.

The toxin may be associated closely with pathogenicity. *H. mammatum* is primarily a pathogen of quaking aspen, although it has been reported on other species in a parasitic or saprophytic role (2, 5, 7). On most species, wounds inoculated with *H. mammatum* rapidly develop callus and no infection occurs. These species also are not sensitive to the *H. mammatum* toxin (Table 4). Of the species showing sensitivity to the toxin, only *P. tremuloides* was highly sensitive; all other *Populus* spp. tested were only slightly sensitive. These same *Populus* spp. were inoculated with *H. mammatum* (5) and were found to be immune or only slightly susceptible to infection and canker development. On some of the species, a canker began to develop, but then healed over.

Of the 14 isolates of various fungi that were tested, only two, *H. mammatum* and *Cenangium singulare*, produced positive necrotic reactions in the aspen leaf bioassay. The *C. singulare* extract produced a reaction that could not be distinguished from that produced by the *H. mammatum* extracts.

Detection of the toxic activity in cankered portions of quaking aspen trees and in the regions immediately adjacent to cankers indicates that the toxin may produce the canker symptoms caused by *H. mammatum*. Although large amounts could be detected within the canker, the toxin reaction was weaker in the 5-cm portion of bark and wood surrounding the canker. Therefore, the toxin must only act near where it is produced and released by the fungus mycelium. This observation could explain why the symptoms of *H. mammatum* infection is a limited necrosis rather than a long necrotic streak such as might occur if the toxin was readily translocated.

Experiments with *H. mammatum* grown in liquid culture show that the toxin is excreted rapidly from the mycelium of the fungus; however, none can be demonstrated in extracts of mycelium. Because the toxin could be demonstrated in cankered host tissue as well as in culture and because the amounts in culture increased with time, the toxin evidently is a regular metabolic product of *H. mammatum* growth rather than a material produced in response to the host.

Gel-filtration experiments indicated that the toxin is a molecule or group of molecules with an approximate molecular weight between 600 and 1,800. The molecular weight of the parent ions of all components detected by mass spectrometry was 1,100 or less. Further experimentation with both silica gel column chromatography and TLC indicated that the toxin might

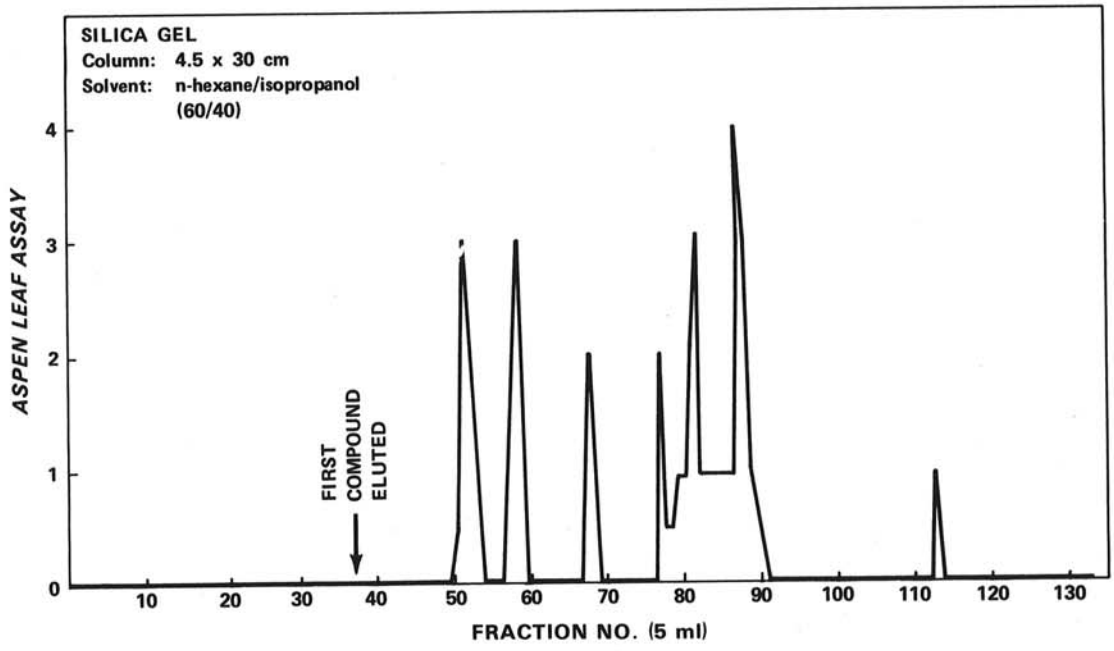

Fig. 4. Silica-gel chromatography of *Hypoxylon mammatum* toxin. The arrow indicates the point at which the first material detectable at 260 nm was eluted. Each fraction was assayed by the aspen leaf bioassay technique.

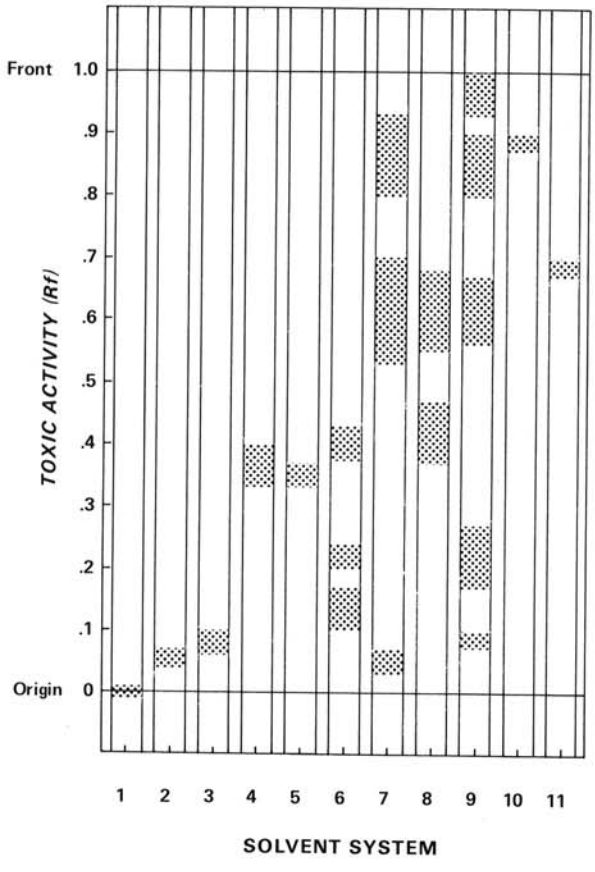

Fig. 5. Diagrammatic representation of thin-layer chromatograms of the toxins extracted from *Hypoxylon mammatum* cultures. Toxic activity in water eluates of the chromatograms are shown by shaded portions of the diagrams. The solvent or mixtures (v/v) used were: 1. toluene; 2. chloroform/methanol (99/1); 3. chloroform/acetone (90/10); 4. chloroform/ethanol (80/20); 5. chloroform/methanol/acetic acid (67/16.5/16.5); 6. benzene/ethanol (80/20); 7. ethyl acetate; 8. ethyl acetate/acetone (90/10); 9. benzene/ethanol/acetic acid (67/16.5/16.5); 10. benzene/methanol/acetic acid (79/14/17); 11. ethanol/water/boric acid/acetic acid (70/26/3/2).

consist of as many as seven components.

Toxin activity was detected in a whitish material that sublimed at a temperature between 65 C and 110 C. No attempt was made to determine at which temperature in that range the toxin sublimed. Because the toxic substance is highly soluble in acetone, water, and alcohols, it appears that it is a hydroxylated compound or group of compounds. The toxic substance is not soluble in nonpolar solvents such as chloroform, ether, or *n*-hexane. In other experiments the toxic substance in crude extract was found to be heat-stable up to at least 121 C.

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