Mammatoxin Assay for Genetic and Environmental Predisposition of Aspen to Cankering by *Hypoxylon mammatum*

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ABSTRACT

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Mammatoxin, a fungal toxin produced by *Hypoxylon mammatum*, was used to measure genetic and environmental effects on susceptibility of aspen (*Populus tremuloides*) to cankering. Potted aspen sprouts of four clones were preconditioned to two levels of moisture and four soil conditions. Mammatoxin lesion size from leaf bioassay was significantly affected by moisture, soils, clones, and moisture-soil-clone interactions. Lesion size was positively correlated with percentage of infection in natural stands. The leaf-toxin assay may be a fast and inexpensive method for determining genetic and site predisposition to cankering.

Genetic and environmental factors play significant roles in the etiology of Hypoxylon canker of trembling aspen (*Populus tremuloides* Michx.) (3,11; R. I. Bruck and P. D. Manion, *unpublished*). Testing for resistance has been based on stem and branch inoculations with mycelia of *Hypoxylon mammatum* (Wahl.) Miller (1,5,11). Mycelial inoculations have been successful in the field (11,17) but unpredictable in assessing canker initiation and elongation in potted sprout experiments (2,10,12). Mammatoxin, a fungal metabolite derived from *H. mammatum* culture filtrates, is reported to be responsible for canker development in aspen (15,16). French (10) used mammatoxin to screen aspen for genetic resistance to cankering, but results of screening *Hypoxylon*-resistant genotypes were inconclusive.

The purpose of our research was to utilize mammatoxin in potted plant experiments to discern the role of aspen clonal genetics, differing environmental regimens, and genetic-environment interactions on the susceptibility of aspen to Hypoxylon canker.

MATERIALS AND METHODS

Plants. Cuttings were propagated from aspen root sprouts taken from four clones selected to reflect a large variation in field cankering susceptibility. The clones were representative of a larger sample of 23 stands intensively investigated by us (*unpublished*) for correlations between environmental variables and canker incidence.

The cuttings were treated with a rooting hormone (Hormo-Root "B", indole-3-butyric acid 4%, thiram 15%, Hortus Products Co., Newfoundland, NJ), planted in perlite and placed under an intermittent misting system. Rooted sprouts were transplanted to sterilized U.C. soil mix (4) in 10-cm plastic pots, fertilized monthly, and watered to field capacity weekly for 1 yr. Sprouts grew to approximately 60 cm. Ortho-Ethion (Ethion Dust 4%, Chevron Chemical Co., Cherry Hill, NJ) was applied weekly for mite control.

Soils. Circular block furrow slices of field soil were collected from each of the four sampling sites of the respective test clones. Care was taken not to disturb the compositional and textural integrity of the samples as they were placed in 38-cm deep, 10-L polyethylene containers. After being cleaned of soil residue and having roots pruned to about 10 cm, four sprouts, one from each clone, were planted in each container. Four replicate containers were similarly planted for each of the four soil samples, yielding 16 experimental containers with a total of 64 sprouts. An additional four containers, each with a single sprout, were used as controls to determine whether interplant competition for available soil nutrients was significant.

The entire experimental block was watered to field capacity every 3 days for 108 days. Samples of leaf lamina were removed from the 16 experimental subsets and assayed for nutrient concentrations spectrophotometrically (21).

Mammatoxin. *Hypoxylon mammatum* was grown on a basal synthetic medium (2) in 2-L flasks on a reciprocal shaker (80 rpm) at 20 C for 14 days. Large mycelial balls were filtered from the basal media, and the resulting filtrate was concentrated to one-tenth original volume by flash evaporation. This crude mammatoxin filtrate was used in the assay experiments.

Assay. After 108 days of predisposition, four leaves from each plant were excised. Leaf petioles were placed in small vials of distilled water. Six puncture wounds were made on each leaf, and a 5-μl drop of crude filtrate was placed on each wound. A similar drop of noninoculated culture medium was placed on four puncture-wounded control leaves. The leaves were incubated at room temperature under a fluorescent light in a humidity chamber (>97% relative humidity). Lesion diameters were measured 18 hr after toxin treatment.

The experimental assay was repeated on the same plants 14 or 15 days later when the plants, which had been deprived of water, began to wilt. The plants were then watered to field capacity every 3 days for 15 days. A second assay of the plants at field capacity was followed by a moisture stress period, and a second wilting point assay was performed on two leaves per plant. A total of 4,896 lesions on 816 leaves were assayed during the experiment.

RESULTS

A 5-μl drop of crude filtrate placed on leaf puncture wounds produced a spreading tan-colored lesion within 1 hr; this was surrounded within 3 hr by a black outer ring (Fig. 1). No lesions were observed on wounded leaves treated with undiluted, noninoculated culture medium.

Environmental predisposition. Significant differences in aspen leaf nutrient concentrations were observed after the 108-day predisposition period (Table 1). Analysis of foliage of greenhouse-grown aspen compared with that of field-grown aspen on the same soils (8) showed correlation coefficients of 0.96 or greater (P = 0.01) for K, Mg, Ca, and Mn. Coefficients of 0.89 or greater (P = 0.05) were calculated for P and Na. Foliage of the four control plants (one tree on its native soil per pot) differed insignificantly for nutrient concentration compared with test plant foliage samples from experimental containers.

With the exception of P, all nutrients examined were lower in greenhouse-grown foliage than in corresponding field tissue. Regular spraying of Ethion resulted in P uptake by all the plants. This P "fertilization" may have distorted the relative differences in the observed nutrient concentrations.

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Mammatoxin assay. Results of toxin bioassay on 16 soil-clone interactions at both field capacity and wilting point moisture regimens are summarized in Table 2. A progression from large to small lesion diameters is apparent. All interactions involving clone 2 (natural field canker incidence of 70%) showed greater lesion diameters than did those involving clone 16 (35% canker incidence), clone 17 (20% canker incidence), or clone 6 (0% canker incidence). Lesion diameters also decreased in a linear manner from soil 2 to soil 6. Without exception, the plants watered to field capacity had smaller lesion diameters than the plants stressed to wilting point.

Results were analyzed with a multi-dimensional analysis of variance test (18). Highly significant differences for the two moisture conditions, the four soil types, the four clones, the three two-way interactions, and the three-way interaction validate the trends shown in Table 2. Nonsignificant differences for the four plants in each clone, the four (or two) leaves on each plant, and the six lesions per leaf demonstrate the consistency and repeatability of the results. All control plants showed insignificantly different mammatoxin lesion diameters ($P = 0.01$) than their experimental counterparts.

DISCUSSION

Host-specific fungal phytotoxins have been successfully used to screen plants for disease resistance (9,19,20). The effectiveness of such assays is reflected in their accuracy, rapidity, and low cost. The results of our research support the use of mammatoxin for such applications.

The black-ringed tan lesion reaction observed in our experiments does not conform to previously reported mammatoxin assays. Stationary flask cultures of our isolates resulted in a toxic extract that produced small black lesions on aspen leaves 3 days after treatment, similar to the observations of French (10). Schipper (14,16) used extracts from grain cultures and recovered a toxin that, when administered through the leaf petiole, produced only black lesions.

The reciprocal shaker-grown cultures always produced both the tan and the black components, which initiated rapidly spreading large lesions in a relatively short time. The speed of this reaction allowed a fast and accurate assay.

The 108-day sprout environmental predisposition period to the potted soil block samples was sufficient to express significant differences in aspen tissue nutrient concentrations. Similar soil block studies have been used in agricultural fertilizer trials and have appeared to be more accurate in testing plant-soil interactions than composite soil mixtures (13). When the greenhouse aspen tissue nutrient concentrations were subjected to a correlation analysis with field canker frequency of the four clones, statistically significant coefficients were realized for all nutrients except P; miticide contamination may have weakened this correlation.

The relationship between canker incidence, foliage nutrient concentrations, and soil nutrient exchange capacities was shown for 23 sample plots, including the four in this study (R. I. Bruck and P. D. Manion, *unpublished*). When tissue concentrations were tested against mammatoxin lesion diameters, highly significant correlations were observed in all cases (Table 3). Soil nutrient exchange capacity, as reflected in tissue uptake of minerals, significantly correlates to both the natural infection rate and the assay of this apparent susceptibility via mammatoxin.

Although French (10), using potted

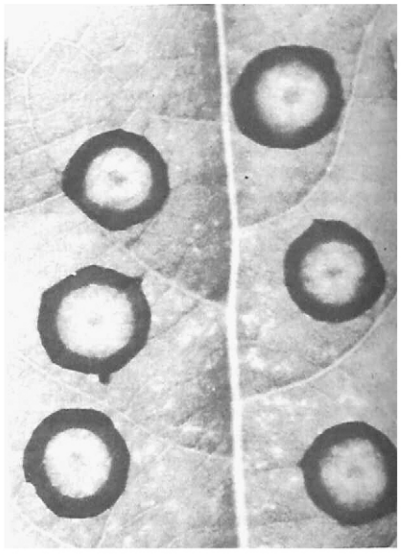

Fig. 1. Black rings around tan lesions induced by a 5-µl drop of *Hypoxylon mammatum* crude filtrate placed on leaf puncture wounds. Assay performed 18 hr after toxin treatment.

Table 1. Concentration of major and minor nutrients as percentage of dry weight of greenhouse-grown aspen sprout foliage predisposed for 108 days to four soil types

Soil number	Nutrient	Clone number 2	16	17	6
2	P	0.400	0.531	0.800	0.611
	K	0.520	0.611	0.655	0.645
	Ca	0.268	0.302	0.281	0.375
	Mg	0.077	0.133	0.113	0.176
	Mn	0.020	0.027	0.030	0.034
	Na	0.010	0.031	0.033	0.037
16	P	0.437	0.683	0.642	0.664
	K	0.431	0.471	0.513	0.520
	Ca	0.278	0.331	0.387	0.402
	Mg	0.122	0.127	0.168	0.198
	Mn	0.027	0.029	0.028	0.039
	Na	0.021	0.038	0.041	0.038
17	P	0.491	0.617	0.658	0.658
	K	0.561	0.584	0.622	0.720
	Ca	0.280	0.354	0.407	0.413
	Mg	0.101	0.153	0.187	0.264
	Mn	0.021	0.033	0.040	0.041
	Na	0.022	0.041	0.047	0.043
6	P	0.513	0.718	0.793	0.821
	K	0.642	0.671	0.712	0.892
	Ca	0.332	0.368	0.413	0.507
	Mg	0.171	0.184	0.292	0.313
	Mn	0.031	0.038	0.042	0.054
	Na	0.027	0.048	0.050	0.064

Table 2. Average diameters (mm) of mammatoxin lesions (induced by applying 5 µl of crude extract) and standard deviation of 16 soil-clone aspen sprout interactions at field capacity (F) and wilting point (W) moisture regimens

Soil number	Moisture	Clone numbers and percent field cankering 2 mm (70%)	16 mm (35%)	17 mm (20%)	6 mm (0%)
2	F	5.91 ± 0.44	5.70 ± 0.36	4.66 ± 0.28	3.93 ± 0.28
	W	6.47 ± 0.68	6.01 ± 0.39	5.37 ± 0.53	4.62 ± 0.41
16	F	5.46 ± 0.31	5.13 ± 0.25	4.23 ± 0.21	3.71 ± 0.27
	W	5.89 ± 0.70	5.61 ± 0.39	5.20 ± 0.63	4.00 ± 0.32
17	F	5.13 ± 0.37	4.88 ± 0.41	3.96 ± 0.17	3.41 ± 0.17
	W	5.88 ± 0.37	5.67 ± 0.62	4.17 ± 0.45	3.77 ± 0.29
6	F	4.81 ± 0.50	3.85 ± 0.37	3.47 ± 0.33	2.62 ± 0.20
	W	5.01 ± 0.48	4.41 ± 0.39	3.99 ± 0.41	2.93 ± 0.21

Table 3. Correlation coefficients of nutrient concentrations of greenhouse-grown aspen sprouts to field *Hypoxylon mammatum* frequency and mammatoxin lesion diameters for four clones of their native soil type

Nutrient	Correlation coefficients	
	H. mammatum frequency	Mammatoxin lesion diameter (mm)
P	− 0.837	− 0.898*
K	− 0.921*	− 0.881*
Mg	− 0.877*	− 0.913*
Ca	− 0.973**	− 0.959**
Mn	− 0.989**	− 0.982**
Na	− 0.879*	− 0.871*

* Significant at 5% level of confidence.
** Significant at 1% level of confidence.

seedlings, found no correlation between toxin lesion diameters and canker elongation, lesion diameters during our experiments were highly correlated to natural clone canker incidence (r = 0.922). The lack of data corroboration may be due in part to the many factors affecting canker enlargement immediately after artificial inoculation.

We observed wide ranges in soil chemical and physical factors in 23 study plots (*unpublished*). Four of those plots were chosen for this study because of their differing soil characteristics and floristic composition (8). Soil 2 was an outwash sand deposit that was very well drained and had a high bulk density and low nutrient exchange capacity. Soil 16 was well-drained glacial till, with little organic matter, exchangeable nutrients, or silt-clay content. Soil 17 was a mature, relatively undisturbed silt-loam but was poorly drained because of an indurated fragipan; this soil had a relatively high cation exchange capacity and silt-clay content. Soil 6 was an undifferentiated organic muck that was very poorly drained and had high exchange capacity, high moisture retention, and very high silt-clay content.

All four aspen clones responded in a similar manner when predisposed to the four soil types. With few exceptions, the clones had lower nutrient concentrations and larger mammatoxin lesions when grown on soil 2 than when grown on soil 16, 17, or 6 (Tables 1 and 3).

Genetic variability among clones also apparently influenced nutrient uptake, as sprouts from different clones responded differently to the same soil type. Clone 2 planted on soil 2 consistently had lower nutrient concentrations and larger lesions than clone 6 planted on soil 6. Clone 2 always had significantly lower nutrient concentrations and larger lesion diameters than the other three clones. Thus, nutrient uptake and lesion size appear to respond to a complex of genetic influences and soil fertility levels.

Leaves from sprouts moisture stressed to wilting point had larger lesion diameters than those from sprouts not moisture stressed. Bagga and Smalley (3) observed larger canker elongation in potted moisture-stressed sprouts than in nonstressed sprouts, which is corroborated by our data. Bier and Rowat (6,7) suggested that infection may occur during the tree's dormant period when infection courts would be available for fungus invasion. Along with our data, the fact that dormant trees have a relatively low bark moisture content (6) lends support to the hypothesis that moisture-stressed individuals are more susceptible to cankering under natural conditions.

Our data, which reflect significant interactions of soil nutrient exchangeabilities, moisture regimens, and clonal genetics, support the proposition that both environmental and genetic factors profoundly influence the etiology of Hypoxylon cankering of aspen. The mammatoxin assay appears to be useful in screening genotypes for natural resistance and in assessing the impact of the environment on the disease. A coordinated effort utilizing mammatoxin as a potential disease screening tool may be desirable.

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