SCREENING ASPEN FOR RESISTANCE TO HYPOXYLON CANKER

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ABSTRACT

SCREENING ASPEN FOR RESISTANCE TO HYPOXYLON CANKER

By

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Two methods of screening aspen (Populus tremuloides and P. grandidentata) for resistance to Hypoxylon canker were tested. These included inoculation of naturally existing clones of aspen with pathogenic isolates of Hypoxylon mammatum, and assay of excised leaves from various host genotypes with host-selective metabolites produced in vitro by the fungus. The inoculation method was evaluated by comparing length of cankers resulting from inoculations with the amount of natural infection in each clone.

I. Pathogenic single spore isolates of H. mammatum were identified by inoculating branches of trees in 4 clones of P. tremuloides with 12 isolates. The two most virulent isolates and one isolate with low virulence were used to inoculate 100 naturally occurring clones of trembling aspen (Populus tremuloides) and 13 clones of bigtooth aspen (P. grandidentata) in 12 geographic areas of Michigan during late April and early May. The resulting cankers were measured 70 days after inoculation, and canker length among clones located within the 12 areas was subjected to analysis of variance. The two highly virulent isolates caused cankers on 95% and 96% of inoculated branches, and one isolate produced significantly longer cankers than the other (mean length 59 vs. 46 mm over 100 clones). Cankers ranged from 12 to 14 mm in length. P. tremuloides clones in northern areas produced significantly shorter cankers than those in some southern areas. Significant differences in

length of cankers among trembling aspen clones in 10 areas were observed. Amount of natural infection of Hypopylon canker in each clone was determined at the same time that cankers were measured; infection level per clone ranged from 0% to 58%. Length of cankers resulting from artificial inoculation was not correlated with amount of natural infection among clones in 11 of the areas. Lack of correlation may be ascribed to absence of suitable levels of natural inoculum within some of the areas, so that genetic susceptibility was not expressed.

Branches of P. grandidentata inoculated with H. mammatum became infected, and developed cankers similar in size to those on P. tremuloides. Large amounts of callus were observed on inoculated branches in some clones. Size of cankers following inoculation also varied among clones of this species, and mean length ranged from 29 to 69 mm after infection with the most pathogenic isolate.

II. H. mammatum produced toxic metabolites in culture which were host-selective. Toxic preparations caused spreading necrotic lesions when applied to puncture wounds on leaves of P. tremuloides. The preparations also affected leaves of P. grandidentata and P. maximowiczii. Leaves of 10 other woody species were not affected.

Toxic metabolites from one isolate of H. mammatum were partially purified, and at least two host-selective components were evident. Lesion diameter was plotted against toxin concentration, using leaves of a sensitive clone of P. tremuloides. The response was linear over a 2,000-fold toxin concentration gradient. Toxic compounds were probably less than 1000 d in molecular weight, and did not lose activity after heating to 120 C. Toxic metabolites were produced under all tested conditions which allowed growth of the fungus.

Isolates of H. mammatum differed in ability to produce host-selective toxic metabolites in culture. Non-pathogenic isolates and those with low pathogenicity in inoculation tests produced the lowest amounts of toxin in culture.

Attempts were made to correlate clonal sensitivity to H. mammatum toxin with susceptibility to infection by the fungus. Leaves from 29 clones of P. tremuloides were assayed with toxin preparations, and stems of young trees in the same clones were inoculated with H. mammatum. In general, sensitivity to toxin in leaf assays was not correlated with length of cankers developing from inoculations with the fungus. The lack of correlation in these tests may be ascribed to the juvenile nature of tissues which were inoculated. Additional attempts to correlate sensitivity to toxin with genetic susceptibility to H. mammatum infection are suggested, because it is not clear whether inoculation with the fungus or tests with its host-selective toxin are good indicators of susceptibility to the disease.

SCREENING ASPEN FOR RESISTANCE TO HYPOXYLON CANKER

By

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GENERAL INTRODUCTION

The importance of aspen (collectively, Populus tremuloides Michx. and P. grandidentata Michx.) to pulpwood industries in the United States and Canada has been well documented (12, 13). Since the early 1950's aspen has been the largest single source of pulpwood in the Lake States. In addition, the importance of aspen in wildlife management has been recognized (6, 11).

Aspen is well suited to 10 to 20 year rotations (9), with rapid, natural regeneration after clearcutting. Aspen regenerating in this manner forms natural clones, which may cover up to 35 acres (3, 17). Clones often intermingle in areas that have been continually cut and regenerated for long periods. Isolated clones may form by the production of suckers from the expanding root system of a single seedling growing in the open. Isolated, naturally occurring clones are easily recognized on the basis of homogeneity of sex, fall coloration, time of leaf abscission, leaf shape and size, and bark appearance, among other characteristics (4). Clones of P. tremuloides and P. grandidentata can be established artificially by use of adventitious shoots from root cuttings of clones or from individual trees (18).

The impact of Hypoxylon canker, caused by Hypoxylon mammatum (Wahl.) Miller (syn. H. pruinatum), on the aspen resource is substantial (1, 14). In Michigan, Wisconsin and Minnesota, the annual loss of 8.4×10^6 m^3 (300×10^6 ft^3) of aspen per year to Hypoxylon canker is greater than

the desirable level of harvest (16). Control of the disease would be of obvious benefit in expanding the production of aspen, but currently there are no practical control measures. "Improved" aspen plantations are being established (5), and there is some evidence that genetic resistance to Hypoxylon canker could be incorporated into stock for these programs (15).

There appear to be differences in susceptibility of clones to natural infection (8). Significant differences were detected in amounts of natural infection among clones growing in areas of homogeneous environment and inoculum density. While identification of resistant genotypes may be practical and easily performed under such conditions, methods of identifying resistance independent of the vagaries of climate and inoculum density might find more widespread application. Two such methods were attempted in the following experiments: clones were inoculated *in situ* with living mycelium of *H. mammatum*; excised leaves from aspen clones were tested for their relative sensitivity to a host-selective toxin produced by the fungus in culture.

There are indications that clones of *P. tremuloides* will differ in length of cankers following inoculation with *H. mammatum* (10). Clone variability was masked, however, because of the extreme variability in pathogenicity of *H. mammatum* isolates (2, 10), and the apparent clone-isolate interactions with some isolates (10). Therefore, *H. mammatum* isolates with high pathogenicity were identified in these experiments which could be relied upon to cause large cankers on many aspen genotypes. These isolates were used to test for differences in rate of canker enlargement among clones, with the premise that clones producing the smallest cankers may be most resistant to the disease. Attempts

were made to correlate the measured length of cankers with amount of natural infection in such clones.

Recent reports (19, 20) of a host-selective toxin produced in culture by H. mammatum indicated its possible use in identifying resistance to the fungus among genotypes of P. tremuloides. Host-selective toxins are used in screening agricultural crops for disease resistance (7, 21, 22). The objectives of this work were 1) to verify reports of the occurrence of a host-selective toxin from H. mammatum and 2) to attempt to determine if relative sensivity of aspen to the toxin is correlated with actual differences in susceptibility to Hypoxylon canker.

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PART I

Inoculation of Aspen with *Hypoxylon mammatum* as a Possible Method of Identifying Resistant Genotypes

INTRODUCTION

Aspen and other poplars are increasingly important raw materials in wood fiber industries. Quaking aspen (Populus tremuloides Michx.) provides 45% by volume of paper and boxboard fiber in the Lake States (5), and is used to a large extent in Canada (10). Hypoxylon canker, caused by Hypoxylon mammatum (Wahl.) Miller (syn. H. pruinatum), is a limiting factor in growth of aspen (1, 12). Genetic improvement of the species may be possible, especially for use in stands with rotation periods of ten to twenty years (4, 7), and such improvement could include resistance to Hypoxylon canker (13). Bigtooth aspen (P. grandidentata Michx.) is virtually immune to natural infection by the fungus, but evidence of resistance in quaking aspen is lacking.

The clonal growth of aspen in nature was described by Barnes (3). Copony (6) indicated that clones differ in amounts of natural infection, but the meaning of this variability in terms of genetic resistance is not clear. The following experiments tested inoculation with living fungus mycelium as a possible technique for screening clones in situ for resistance to H. mammatum, and attempts were made to correlate the size of the resulting cankers with amount of natural infection in the clones.

MATERIALS AND METHODS

Preparation of inoculum. Single ascospore isolates of H. mammatum were obtained from perithecial stromata on a naturally-infected tree in Ingham County, Michigan. All eight spores were removed from a single ascus in serial order, and cultured on 2% malt extract agar. Nine additional single spore isolates with known pathogenicity were obtained from New York State. These latter isolates had been used in previous tests of artificial inoculation (9), and exhibited wide variability in morphology and virulence. All cultures were maintained separately on 2% malt extract agar.

Three weeks before use for inoculation, a mycelial suspension was prepared from each isolate culture by aseptically blending a portion of the culture in sterile water. One ml of each suspension was introduced aseptically into 20 ml screw-cap vials half filled with moist, sterile wheat. Wheat grains were colonized rapidly, and were shaken periodically to insure complete colonization. Preliminary tests using both infested wheat grains and infested agar plugs as inoculum indicated that infested grain produced more and larger cankers than did agar plugs.

Inoculations in 1974.-Four P. tremuloides stands near East Lansing, Michigan were selected. These were determined to be natural clones based on root continuity between ramets, homogeneity of leaf morphology, fall coloration, and other similarities (3). Two of the clones were growing on well-drained sites, and two on poorly drained sites. All four clones were gynecious.

During June, July and August, a total of 39 trees (4-10 cm DBH) on the periphery of each clone, were incoulated with 12 H. mammatum isolates as described in previous work (9). Thirteen trees were inoculated during each month. Each tree was inoculated on four individual branches with one of four isolates (or 3 isolates plus a control wound). Branches were about 2 m from the ground, and inoculations were made 6-10 cm from the main stem at the side of each branch. A 4 mm diam wound was cut through the bark which exposed the xylem. Each wound was inoculated with a single wheat grain infested with the appropriate isolate. Control wounds received a moist, sterile wheat grain. Wounds were covered with 2 cm wide masking tape, wrapped twice around the branch. Isolates were grouped in incomplete block fashion, using trees as blocks. Special care was taken to distribute the four replicate inoculations evenly around the somewhat circular area occupied by each clone.

Canker lengths were measured 70 days after inoculation, and data were subjected to analysis of variance with canker length as the dependent variable.

Inoculations in 1975.-One-hundred natural clones of P. tremuloides, occurring in 12 geographic areas of Michigan (Fig. 1), and 13 clones of P. grandidentata in 7 of these areas, were inoculated as described above in late April through early May, except that wounds were covered with 3 cm wide Parafilm M (American Can Co.) in 1975 tests, instead of 2 cm wide masking tape as in previous experiments (9, 15). Clones were inoculated from south to north, coincident with spring leaf emergence, to reduce variability caused by differing phenology of the plants. Two isolates with high virulence and one isolate with low virulence in 1974

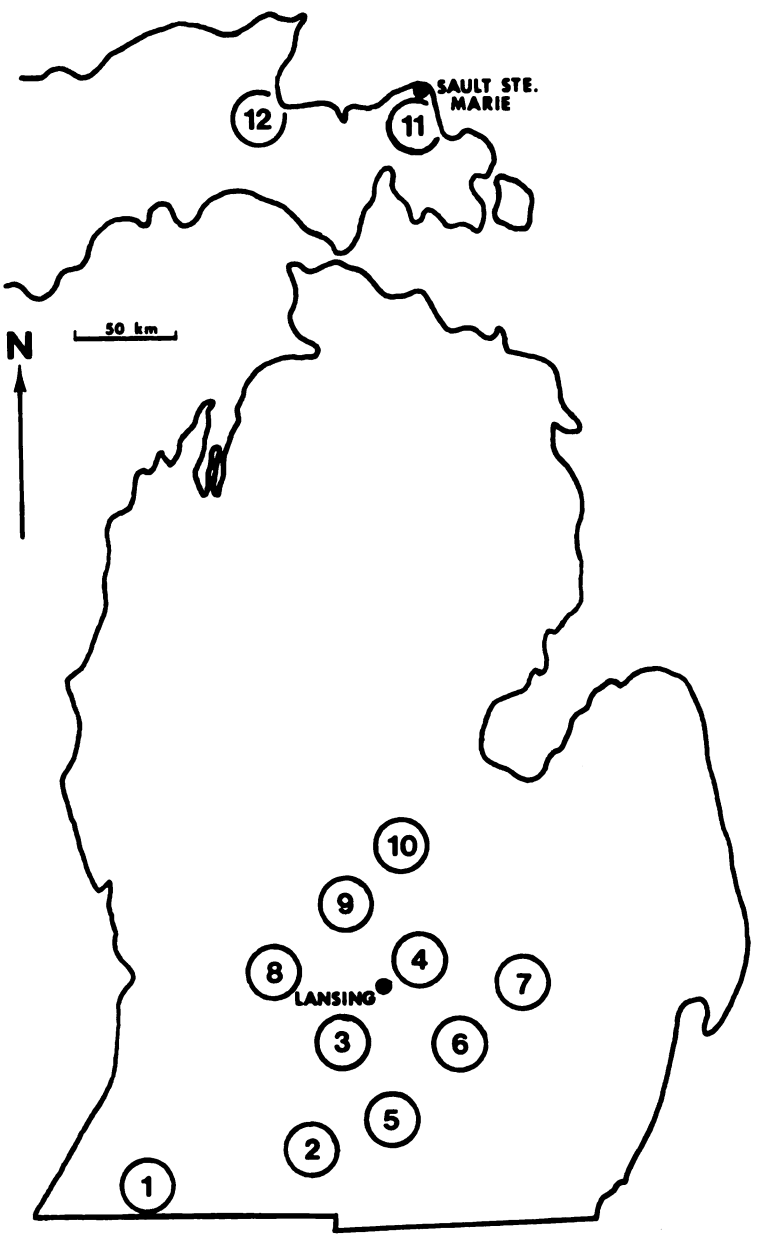

Figure 1. Locations of 12 geographical areas of Michigan wherein aspen clones were studied during 1975. Each area is approximately 20 km in diameter. Seven to 10 clones were located in each area.

tests, and one control inoculation, were used on 4 separate branches of 5 replicate trees in each clone. Canker were measured 70 days after inoculation.

Survey of natural infection.-The number of naturally infected trees in each of the clones inoculated in 1975 was counted at the same time that cankers were measured. All trees examined were 4 cm DBH or greater, and were termed infected if one or more cankers on the main stem were visible from the ground. All trees in most clones were examined. In clones with more than 100 trees, a transect 4 m wide was made through the center of each clone so that the ortet was included. Dead trees were counted as infected if H. mammatum stromal masses were clearly visible.

RESULTS

Inoculations in 1974. Cankers resulting from artificial inoculation became visible outside their masking tape covers in about 6 weeks. Cankers extended rapidly in basal and apical directions from the wound. By 10 weeks after inoculation cankered bark was wrinkled and cracked, with a typically yellow-orange mottled surface. Excision of outer bark indicated more extensive necrosis and discoloration beneath the surface which included the xylem near the inoculation wound. Some branches were completely girdled by necrotic tissue. The lengths of cankers were measured from apical to basal ends, after removal of outer bark. Mean lengths of cankers caused by inoculation with 12 isolates during June, July, and August are listed in Appendix A.

Four of the 12 isolates were generally non-pathogenic, producing short cankers (21 mm or less) on 0-8% of inoculated branches. Five isolates produced cankers after inoculation in all 3 months of the experiment, while 3 isolates produced cankers only following June and July inoculations. Success of inoculations in August was poor and the smallest cankers were observed after August inoculations. Only 6 of the 12 isolates produced cankers on all 4 clones during June and July. Statistical analysis was restricted to data obtained with these 6 isolates, from inoculations made during June and July.

Analysis of variance of canker length produced by the six isolates indicated a significant effect of time of inoculation (Table 1). The

Table 1. Analysis of variance of canker length caused by inoculation of 4 P. tremuloides clones with 6 isolates of H. mammatum during June and July, 1974.

Effect	Degrees of Freedom	Mean Square	F significant at P =
Month	1	3345.19	.005 **
Isolate	5	2814.45	.001 **
Clone	3	5356.45	.001 **
Clone within isolate A	3	1851.96	.005 **
Clone " " B	3	1196.56	.035 *
Clone " " C	3	1160.62	.039 *
Clone " " D	3	1138.59	.042 *
Clone " " E	3	925.80	.081
Clone " " F	3	869.10	.097
Clone X Month	3	638.56	.196
Isolate X Month	5	135.48	.890
Isolate X Clone	15	296.35	.737
Isolate X Clone X Month	15	189.99	.473
Error	112	401.88 a/	

a/ Error mean square = s^2, where s = standard deviation of canker length within a clone; s = 20.0 mm.

** indicates highly significant effect

* indicates significant effect

longest cankers resulted from June inoculations. Clone and isolate effects on canker length were also significant. No two-way or three-way effects were significant at $P<.1$.

Additional partitioning of the clone sum of squares within isolates identified those isolates which produced the significant clone effect. Of these, the isolates producing the most significant clone effects (isolates A and B, Table 1) also produced the most cankers (41 and 44 cankers respectively on 48 total inoculated branches), and the longest cankers (mean length of 92 mm and 72 mm respectively, on clone RL 11 inoculated in June). The isolate with lowest virulence caused one short canker on 48 inoculated branches in 1974. This isolate (Appendix A, isolate 605-6) had caused few cankers in previous tests (9). Isolates A and B were selected as having the highest virulence, and isolate 605-6 having the lowest virulence; hence they were used in the 1975 tests.

Inoculation of clones in 1975.- Cankers appeared sooner in 1975 tests, emerging from the paraffin film cover after about 4 weeks. The two pathogenic isolates caused cankers on 465 (94.5%) and 472 (96.1%) of 492 and 491 branches of P. tremuloides, respectively. Cankers ranged from 12 to 114 mm in length 70 days after inoculation. The isolates with low virulence caused 13 cankers on 565 inoculated branches (2.3%), while control wounds produced 4 typical Hypoxylon cankers on 520 branches of P. tremuloides. Mean lengths of cankers produced by the two virulent isolates on 100 clones of P. tremuloides are listed in Appendix B.

Canker lengths produced by the two highly virulent isolates were subjected to analysis of variance, using the sum of canker length produced by the two isolates on each tree as the dependent variable

(Appendix B). Among the P. tremuloides clones, a significant area effect was indicated (Table 2). Clones in northern areas (Fig. 1, areas 11 and 12) had significantly shorter cankers than those in area 8. Clones within areas were also significantly different, with some clones producing longer cankers than others. Additional partitioning of sums of squares identified those areas in which clones were significantly different. Clones within 10 of the 12 areas produced cankers of different lengths after 70 days (error probability <.05).

The two highly virulent isolates produced cankers of different size, indicated by the significant isolate effect. Isolate A usually produced larger cankers than isolate B (overall mean length of 58.5 mm and 45.7 mm respectively). This difference was statistically variable among regions, however, as the isolate X region effect was significant (Table 2).

Amount of natural infection in clones of P. tremuloides ranged from 0% of the trees infected within 14 clones, to 58% in the clone with heaviest infection. Regression analysis was applied to 99 of the 100 clones, with 20 to 100 trees in each. Percent of naturally infected trees in each clone was plotted as the dependent variable against each of three independent variables: 1) mean canker length (MCL) produced by isolate A on the clone; 2) MCL produced by isolate B on the clone; 3) MCL produced by isolate A plus MCL produced by isolate B on the clone. Significant linear correlations between lengths of cankers resulting from inoculations and amount of natural infection were not observed in 11 of the 12 areas, using any of the 3 variables.

Branches of P. grandidentata inoculated with isolates A and B became infected, and produced cankers that were 13 to 99 mm long 70 days

Table 2. Analysis of variance of canker length caused by inoculation of 100 P. tremuloides clones occurring in 12 geographic areas of Michigan. Clones were incoulated with 2 pathogenic isolates of H. mammatum in April-May, 1975.

Effect	Degrees of Freedom	Mean Square	F significant at P =
Areas	11	11094.94	.001 **
Clones (Areas)	88	1396.82	.001 **
Clones (Area 1)	5	1774.77	.001 **
" (Area 2)	7	1878.73	.001 **
" (Area 3)	9	618.23	.111
" (Area 4)	6	852.44	.041 *
" (Area 5)	7	2663.17	.001 **
" (Area 6)	9	1766.05	.001 **
" (Area 7)	8	1429.14	.001 **
" (Area 8)	7	1631.89	.001 **
" (Area 9)	5	1946.26	.001 **
" (Area 10)	9	938.01	.011 *
" (Area 11)	9	486.41	.254
" (Area 12)	7	1467.53	.001 **
Trees [Clones (Areas)] (Error)	346	384.09 a/	
Isolates	1	53482.16	.001 **
Isolates X Areas	11	718.49	.016 *
Isolates X Clones (Areas)	88	359.23	.313
Isolates X Trees [Clones (Areas)] (Error)	346	332.79	

a/ Error mean square = s^2, where s = standard deviation of canker length within a clone; s = 19.6 mm

** indicates highly significant effect

* indicates significant effect

after inoculation. Isolates A and B produced cankers on 59 (95%) and 60 (97%) of 62 and 63 inoculated branches respectively. Cankers in some clones were surrounded by large amounts of callus tissue. Analysis of variance of canker length among clones, disregarding possible geographic area effects, indicated significant clone differences in reaction to both highly virulent isolates (Table 3). At least 15 trees in each clone of P. grandidentata were examined, and only one naturally infected tree was found.

Table 3. Analysis of variance of canker length caused by inoculation of 13 P. grandidentata clones with 2 isolates of H. mammatum. Clones were located in 7 geographic areas of Michigan, but were analyzed disregarding possible area effects.

Effect	Degrees of Freedom	Mean Square	F significant at P less than
Isolate A:			
Clone	12	12518.99	.01
Error	46	8041.50	
Isolate B:			
Clone	12	69860.70	.01
Error	47	3188.30	

DISCUSSION

Previous work with H. mammatum demonstrated extreme variability in virulence of isolates (2, 9). Genetic variation in susceptibility of the host was also suggested among clones of P. tremuloides (6, 9), and among interspecific hybrids of P. tremuloides and P. grandidentata (13, 15). Infection in nature is most often associated with young lateral branches near the main stem (8, 11), or with insect galls on branches (11, 14). Therefore, lateral branches of young trees were inoculated in an attempt to measure the variation in susceptibility among clones.

The experiment in 1974 demonstrated that inoculations in June produced more and larger cankers than later inoculations. In addition, use of H. mammatum isolates with high virulence contributed to production of cankers, with successful infections on 95-96% of inoculated branches in these tests. A single ascospore isolate, representing a single genome of the fungus, may be more reliable for producing cankers on many host genotypes than isolates from margins of natural cankers ("mass" isolates) which are of unknown nuclear condition (16).

Differences among clones in length of cankers 70 days after inoculation were observed in 1974 and 1975. The concurrent observation of differences among geographical areas might indicate a macroclimatic control of canker size. However, the significant differences among clones within 10 of the 12 areas (Table 2) would more clearly indicate

either microclimatic or genetic control of canker length. In addition, within-clone variance of canker length was sufficiently small (s = 20.0 mm in 1974; s = 19.6 mm in 1975, Tables 1 and 2), to indicate highly significant clone differences, with probability of error less than .01 in most areas.

The meaning of this variability among clones as it relates to actual resistance or susceptibility to H. mammatum could be questioned. Length of cankers following artificial inoculation was generally not correlated with amount of natural infection in the clones. However, infection in nature is subject to external variables which include inoculum density and environmental factors. For example, among the seven clones in area 9, 0% to 1% of the trees were naturally infected. This low level of infection may have been the result of low incidence of P. tremuloides, and therefore low amounts of inoculum. Artificial inoculation of these clones, however, produced cankers in frequency and size comparable to those observed in regions with greater amounts of natural infection. Amount of natural infection in a clone, therefore, may be a poor indicator of genetic potential for resistance or susceptibility when determined in areas of differing inoculum potential.

Infection of P. grandidentata branches was observed after inoculation with H. mammatum. Rogers (15) also reported infection of P. grandidentata main stems by similar methods. Concurrent observations in this test of large amounts of callus tissue around the diseased area in branches of some clones may explain the low natural incidence of Hypoxylon canker among bigtooth aspen. The pathogen could be excluded from healthy tissue by this mechanism (15). Clones within this species may differ also in canker length after 70 days, but verification of this

must await further tests on a larger sample of clones under a common environment.

P. tremuloides clones used in these tests were on "average" sites, that is, not in standing water or on particularly dry sites. However, microclimatic and nutritional differences among clones within geographic areas might directly affect length of cankers on these clones. Therefore, artificial inoculation tests such as these may be most useful for identifying potentially desirable host genotypes in the field. Such genotypes could be screened more intensively under a common environment after vegetative propagation. Such propagation is time consuming and expensive, and therefore preliminary screening of clones in situ by inoculation or other means would be economically desirable.

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PART II

Variation of Aspen in Sensitivity to a Host-selective Toxin Produced by *Hypoxylon mammatum*

INTRODUCTION

In 1973 Schipper (15) reported a host-selective toxin produced by Hypoxylon mammatum (Wahl.) Miller. This fungal metabolite had its greatest effect on the major host of the fungus, Populus tremuloides Michx. (trembling aspen), and a lesser effect on P. grandidentata (bigtooth aspen) and P. balsamifera (balsam poplar), which are infrequently attacked species. Low sensitivity was also reported for P. alba (European white poplar) and P. deltoides (eastern cottonwood), which are not infected in nature. Substances toxic to aspen leaves were also found in bark of cankered aspen.

Other host-selective toxins have been used to screen agricultural crops for resistance to the fungi which produce them (4, 18, 19). Therefore, an attempt was made to produce the toxin reported by Schipper, to delineate conditions for its production and purification, and to evaluate its usefulness in screening aspen for resistance to Hypoxylon canker. Clonally-propagated P. tremuloides was used to evaluate differential sensitivity of aspen clones to H. mammatum toxin.

MATERIALS AND METHODS

Host material.- Five clones of P. tremuloides were propagated from root cuttings of natural clones, which were located near East Lansing, Michigan (clones no. 1 - 5). Four clones were gynecious and one was androecious. In addition, four clones of P. deltoides were established from branch cuttings of four trees growing on the property of Michigan State University at East Lansing. Trees were maintained for three years in pots in the greenhouse, and leaf and stem material from these trees were used routinely throughout the present experiments. In addition, 24 P. tremuloides and 3 P. grandidentata clones, propagated independently by Dr. J. W. Hanover, Department of Forestry at Michigan State University, were assayed for their sensitivity to H. mammatum toxin and for their susceptibility to the fungus. Additional leaves collected in the field from P. tremuloides, P. deltoides, P. grandidentata, and P. alba were used.

Pathogen isolates.- A single ascus of H. mammatum was dissected and all eight spores were cultured independently (isolates RL5-1 through RL5-8). Ten additional single ascospore isolates were obtained from New York State. Three "mass" isolates from margins of natural cankers were used in some tests. All isolates had been used previously (6, 7), and hence their pathogenic capabilities were known. In most assays of toxin activity, culture filtrates were used from the isolate with the highest pathogenicity in field tests [isolate RL5-2 (ref. 5, isolate A)].

Production of toxin.- Isolates of H. mammatum were grown in liquid culture media of two types. Fries' liquid medium with yeast extract, used by previous workers (9, 13) was not used due to its toxicity to P. tremuloides tissue. The toxicity was observed after autoclaving the medium. The medium (1) used most often consisted of the following ingredients (in g/liter): glucose 10.0, K_2HPO_4 0.75, KH_2PO_4 0.75, $MgSO_4 \cdot 7 H_2O$ 0.5, $CaCl_2$ 0.1; also present were 1.0 ml of a vitamin stock solution per liter (thiamine, 10.0 mg, plus biotin, 0.5 mg in 100 ml water), and 1.0 ml of a trace element stock solution per liter ($FeC_6H_5O_7 \cdot 3 H_2O$, 214.3 mg; $ZnSO_4 \cdot 7 H_2O$, 158.4 mg; $CuSO_4 \cdot 5 H_2O$, 31.6 mg; $MnSO_4 \cdot 4 H_2O$, 16.2 mg; H_3BO_3, 11.4 mg; MoO_3, 7.0 mg; all in 400 ml water). The medium was adjusted to pH 6.0 with 20% phosphoric acid before autoclaving in 1-liter Roux bottles with plastic foam stoppers, using either 200 or 400 ml of medium per bottle. After autoclaving, a 19.8% solution of L-asparagine was added aseptically to the medium by filtering through a membrane (0.22 μm pores), to provide a final concentration of 0.5 g/liter. Nutritional requirements of H. mammatum for asparagine, thiamine, and biotin were established by Oshima (12). This medium showed no toxicity to leaves of either P. tremuloides, P. deltoides, or P. grandidentata. One ml of a mycelial suspension (containing 3 - 5 x 10^4 hyphal fragments/ml) of H. mammatum was added to each bottle. Cultures were maintained for varying time periods under low light intensity and without agitation in a 27 C room during most tests. Tests of growth and toxin production at different temperatures were performed in incubators, in darkness.

Cultures were filtered through tared, oven-dry, glass-fiber filter paper after incubation. Mycelial dry weight was determined for each culture after oven-drying the residue (90 C, 24 hr) and subtracting the tare weight of the paper.

Purification and extraction of toxin.- After vacuum distillation to 10% or 1% of the original volume, compounds of high molecular weight (above ca. 1000 d) were routinely removed from culture filtrates. Removal was accomplished either by adding an equal volume of cold (-10 C), anhydrous methanol to the concentrated filtrate (14), or by Sephadex G-15 dextran gel filtration with removal of compounds eluting in the void volume, or by a combination of both methods. Fractions containing these large compounds were not toxic to either aspen or cottonwood leaves.

In some instances toxic metabolites were purified further by partitioning into organic solvents, followed by vacuum removal of the solvent and re-dissolution into water. All vacuum evaporation was performed at less than 60 C. Toxin activity was assayed using either crude culture filtrates, deproteinized filtrates, fractions from dextran gel columns, or organic solvent extracts of deproteinized culture filtrates.

Bioassay.- Toxin preparations were assayed by a leaf puncture technique, similar to that used by Hiroe, et al. (8). Excised leaves were punctured with a 16-gauge hypodermic needle, and a 20 µliter drop of test solution was immediately placed on the puncture. Leaves often were treated with four test solutions placed individually in the four quadrants of each leaf. Normally, two to four replicate leaves were used in each assay of any toxin preparation. Leaves were maintained in enclosed chambers (Figure 1), containing distilled water to maintain high relative humidity. Lesions developing from toxic solutions typically radiated from the point of toxin application, producing a discrete black necrotic spot, clearly delineated from the remaining

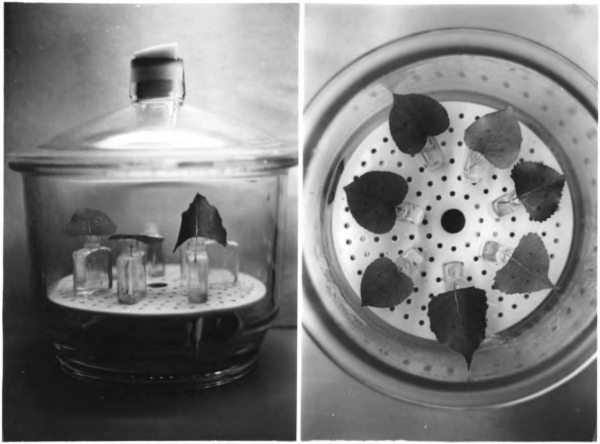

Figure 1 (A and B). Humid chambers in which leaves were assayed with preparations of Hypoxylon mammatum toxin. A) Side view of a converted desiccator, containing distilled water below the porcelain plate. Leaf petioles were immersed in vials containing distilled water, and leaf blades were treated with toxin preparations. B) Top view with cover removed.

green tissue. Lesions were measured at their greatest diameter 24 or 48 hours after beginning the assays. All assays were performed with leaves exposed to fluorescent light at room temperature (21-25 C) in humid chambers.

RESULTS

Host selectivity of toxic metabolites.- H. mammatum isolate RL5-2, a highly pathogenic isolate in field inoculation tests, produced toxic metabolites in culture. These metabolites were host selective, and affected P. tremuloides by producing a spreading, necrotic lesion on excised leaves (Figure 2). Culture filtrates of isolate RL5-2 were active at up to 10-fold dilutions from original concentration, when assayed on leaves from two clones of P. tremuloides (clones 3 and 5), but activity on the other three aspen clones required concentration of filtrates up to 100-fold. No lesions were observed on P. deltoides leaves even after treatment with filtrates that were concentrated 150-fold. In addition, assay of preparations toxic to the most sensitive P. tremuloides clone (clone 5, from which isolate RL5-2 was originally obtained) indicated no sensitivity in the following species, which are not hosts of H. mammatum: P. alba, P. nigra, Salix babylonica, Betula populifolia, Acer rubrum, Acer platanoides, Quercus robur, Tilia sp., and Zelkova sp. (Table 1). Leaves of these species were obtained from nearby ornamentals, and were co-assayed with field-grown leaves of the sensitive clone of P. tremuloides. Tests using intact leaves of P. tremuloides clone 3 and P. deltoides also indicated host-selectivity of toxic metabolites.

Some sensitivity to H. mammatum metabolites was observed with one genotype of P. maximowiczii, which is not known to be infected with H.

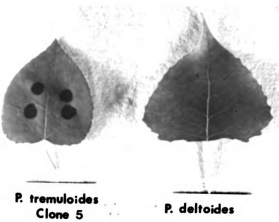

Figure 2. Host-selective toxicity of culture filtrates from Hypoxylon mammatum isolate RL5-2. Each leaf was treated with crude filtrate at four positions, and only the P. tremuloides leaf developed spreading, necrotic lesions.

Table 1. Sensitivity of various woody species to deproteinized filtrates of *Hypoxylon mammatum* cultures.

Species	Sensitivity
Populus tremuloides	high a/
P. grandidentata	low
P. maximowiczii	low
P. nigra	-
P. deltoides	-
P. alba	-
Salix babylonica	-
Betula populifolia	-
Acer rubrum	-
A. platanoides	-
Tilia sp.	-
Quercus robur	-
Zelkova sp.	-

a/ High sensitivity indicates formation of large, spreading, black lesions on leaves after 48 hr; low sensitivity indicates small, brown or black lesions; (-) indicates no necrotic lesions formed.

mammatum in nature. Also, P. grandidentata, which is rarely infected in nature, developed small (<3 mm diam), brown necrotic lesions in assays of leaves from three clones, in contrast to the large, black lesions observed on P. tremuloides leaves.

Physical properties and purification of toxic metabolites.- The toxic portion of H. mammatum culture filtrates eluted in a wide band through columns of Sephadex G-10 and G-15 molecular sizing gels, and was retarded by the gel. Toxic fractions emerged from the gel beginning around 25% of the volume between the void volume of the gel and the end-point of total gel volume (Figure 3). High molecular weight compounds eluting in the void volume were not toxic to P. tremuloides leaves. Also, deproteinization of the filtrates with methanol did not degrade toxic activity.

Toxic culture filtrates were divided into 50 ml aliquots and equilibrated at temperatures ranging from 50 C to 100 C. One aliquot was autoclaved (120 C, 15 min.). All aliquots remained toxic to leaves of P. tremuloides, and did not affect P. deltoides leaves.

Toxin preparations were active on leaves of all ages from sensitive clones, but produced the largest lestions with the most rapid response on young leaves, 5 to 15 cm from the apical meristem. Therefore, only young succulent leaves were used in most assays.

Toxic components were differentially soluble in n-butanol when partitioned against the aqueous filtrate. Toxin was also soluble in acetone, but was not detected in ethyl acetate or chloroform extracts of culture filtrates.

Purification of 70-day culture filtrates from isolate RL5-2 was accomplished by passing the concentrated, deproteinized filtrate through

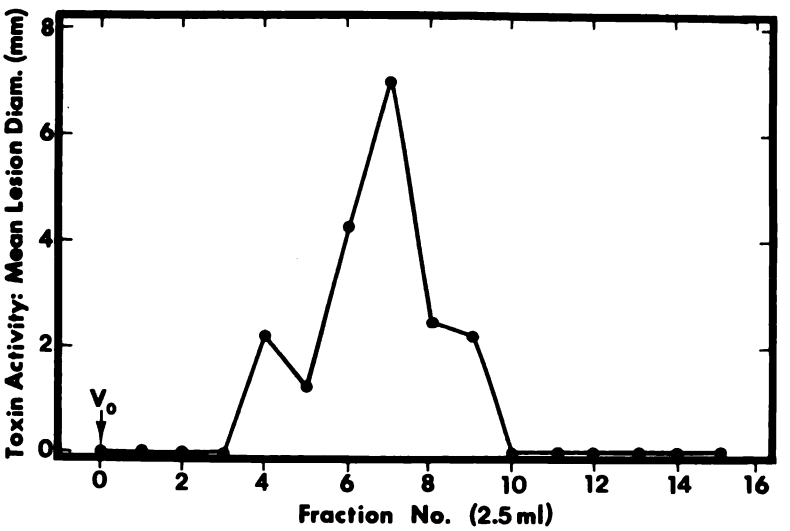

Figure 3. Migration of toxic components through Sephadex G-10 dextran gel. Filtrate from cultures of H. mammatum isolate RL5-2 was concentrated 50-fold; 1.0 ml was applied to the gel (1.5 x 25 cm column) and eluted with distilled water. Each point represents the mean diameter of 2 lesions produced by assaying each fraction on leaves of P. tremuloides clone 5. Compounds eluting in the void volume (V_o) were not toxic.

a large Sephadex G-15 column (2.5 x 30 cm) in two 15 ml aliquots, eluted with distilled water. After assaying and discarding void volume fractions, the low molecular weight fractions were combined and the volume reduced by vacuum evaporation to 21 ml. Fourteen ml of this preparation was partitioned 3 times against an equal volume of water-saturated n-butanol. Assays of the water and butanol fractions after removal of solvents and redissolving in water, indicated that almost all toxic activity was retained by the butanol fraction.

The dried butanol fraction was redissolved in 14 ml of water, and 1.0 ml was applied to a small Sephadex G-15 column (1.5 x 25 cm) and eluted with distilled water. Fractions of 2.5 ml were collected and assayed for toxicity. Toxic fractions were combined, frozen in a 50 ml flask over dry ice, and lyophilized for 24 hr. This procedure yielded a white, fluffy material, which upon microscopic examination appeared comprised of anastomosing filamentous particles. Exposure of this material to air caused it to change into a brown, sticky substance. Assays of such material after redissolving into water indicated no appreciable loss of toxicity.

The final dextran gel fractionation and lyophilization was repeated with another 1.0 ml of butanol soluble fraction in water. When the lyophilized product was flushed with nitrogen, stoppered, and maintained at -15 C, no change in color or form was observed for up to 3 weeks. This preparation was termed lyophilized toxin. The yield of lyophilized toxin was 14.9 mg by this procedure, indicating a concentration of at least 100 mg/liter in the original culture filtrate.

Lyophilized toxin was dissolved in acetone and spotted on silica gel (250 μm thickness) fluorescing thin layer plates. Plates had been

pre-washed with butanol-acetic acid-water (BAW, 3:1:1, v:v:v). Plates were eluted in a single direction with BAW using redistilled solvents. After evaporation of solvent, plates were observed under ultraviolet light and subjected to various spray reagents. At least five compounds were observed with this procedure (Table 2). Gel was scraped from plates in succeeding 1 cm fractions, and compounds eluted into 2.0 ml distilled water. Assays of these fractions indicated that at least two compounds were toxic to P. tremuloides leaves (Table 2, spots 4 and 5).

Lyophilized toxin was dissolved in distilled water at 5.6 mg/ml and diluted serially to 2.8 μg/ml. Each dilution was assayed on P. tremuloides and P. deltoides leaves (Figure 4). A semi-logarithmic plot of lesion diameter vs. toxin concentration indicated a linear dosage response curve with P. tremuloides leaves. P. deltoides leaves were not affected by lyophilized toxin at the concentrations tested.

Production of toxic metabolites under various conditions.- Toxic activity appeared as early as 14 days after initiation of cultures; however, since the fungus is slow-growing in liquid medium, no peak in mycelial dry weight or toxic activity was observed in cultures up to 42 days old (Figure 5). The fungus grew more rapidly in media containing glucose at 5.0 g/liter than at 10.0 g/liter, so 5.0 g glucose/liter was used in later tests of growth and toxin production.

Cultures were initiated in media at pH levels from 3.0 to 7.0, and grown for 24 days. No growth was observed at pH 3.0 (Figure 6). Optimum initial pH for growth of the fungus was 5.0. Toxin was produced equally at all pH levels from 4.0 to 7.0. The fungus buffered the medium toward pH 6.0, but changed the pH very slightly from the initial

Table 2. Compounds visible after thin layer chromatography of lyophilized toxin from Hypoxylon mammatum spotted on silica gel thin layer plates and eluted with butanol-acetic acid-water (3:1:1, v:v:v).

Spot	R_f	Toxin Activity a/	Visible Appearance After Treatment with b/				
			Long UV	Short UV	DSA	DSA + NH_4OH	PNA
1	0.29-0.30	-	blue	-	-	-	-
2	0.39-0.41	-	blue	-	-	-	-
3	0.47-0.53	-	-	dark blue	-	tan (disappears)	red
4	0.64-0.81	+	blue	dark blue	-	-	-
5	0.81-0.96	+	green	dark blue	tan-red	tan	pink

a/ Activity was determined by treatment of leaves of P. tremuloides clone 5 with compounds eluting from each spot in water (+ indicates lesions formed; - indicates no lesions).

b/ Colors are indicated where observed. (-) indicates no visibility or no reaction. DSA (diazotized sulfanilic acid) and PNA (diazotized p-nitroaniline) were freshly prepared (3) and used as spray reagents on separate plates. DSA + NH_4OH treatment indicates that plates sprayed with DSA were exposed to ammonia vapors and then observed.

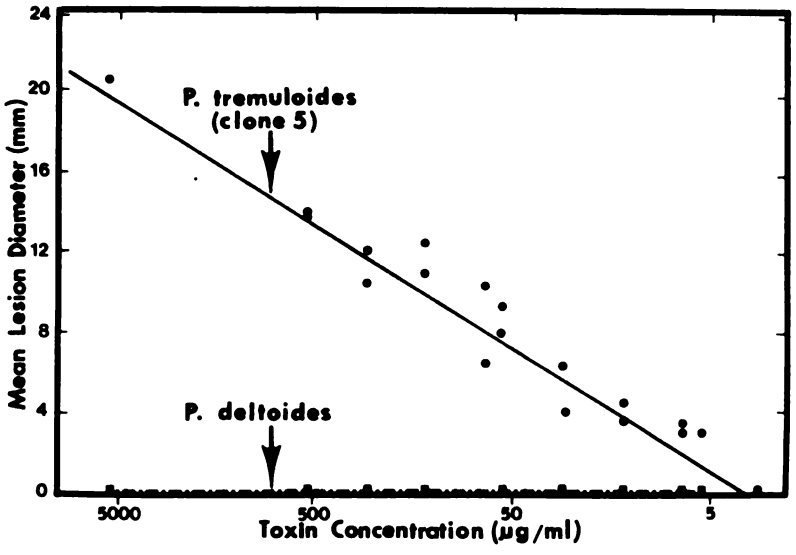

Figure 4. Sensitivity of leaves of Populus tremuloides clone 5 and of P. deltoides to varying concentration of lyophilized H. mammatum toxin. Each point represents the mean diameter of lesions on 4 replicate leaves of each species. The linear regression line indicating response of P. tremuloides leaves was fitted to points by linear transformation of the horizontal axis.

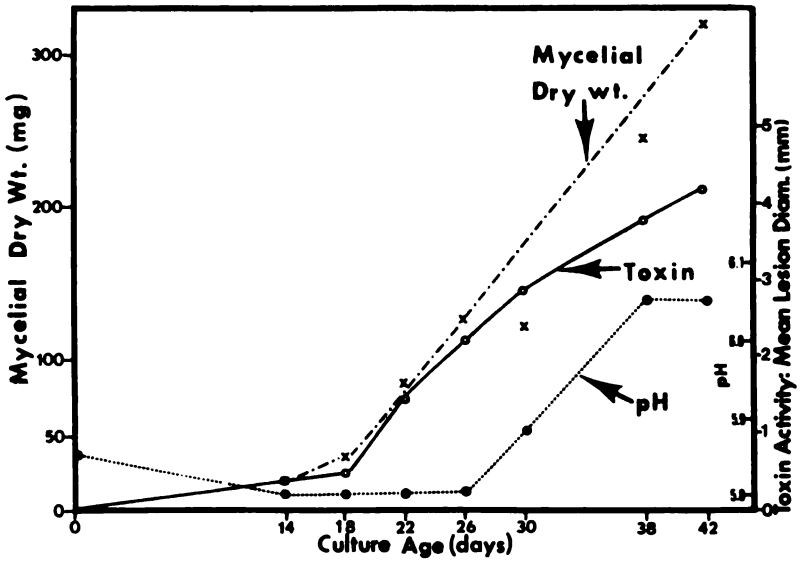

Figure 5. Mycelial dry weight, toxin production, and pH of H. mammatum in cultures incubated up to 42 days. Culture filtrates were all harvested and assayed at the same time. Each point represents mean value obtained from 2 replicate cultures.

pH level, except at pH 4.0. The activity of toxin was greatest at pH 4.0, when filtrates from cultures initiated at pH 6.0 were altered in pH from 3.0 to 10.0.

Cultures were incubated at several temperatures from 15 C to 35 C, using media containing glucose at either 2.5 g/liter or 5.0 g/liter. The fungus did not grow at temperatures above 30 C, and growth at 15 C was barely detectable (Figure 7). Optimum growth for H. mammatum isolate RL5-2 was at 25 and 27.5 C in medium containing 5.0 g glucose/liter. Approximately equal amounts of toxin were produced under all conditions which allowed fungal growth.

Comparative toxin production by fungal isolates.- Five isolates were grown in medium containing 5.0 g glucose/liter. Two highly virulent isolates (RL5-2, RL5-7) one with intermediate virulence (211-8), and two with low virulence (605-6 and RL5-8) (5), were chosen. Inoculum was standardized for each isolate culture to 3×10^4 hyphal fragments per culture bottle. Four replicate cultures of each isolate were incubated for 28 days at 27 C. Each culture was filtered and the residue was weighed; all filtrates from the same isolate were then pooled. To compensate for slow growth and low levels of toxin production by the isolates with low virulence, the volume of pooled filtrate from the 4 slowest growing isolates was concentrated to equal volume per mg dry weight of mycelium. The mean weight of mycelium produced by isolate RL5-7 (the most rapidly growing isolate) was used as a standard. Pooled filtrates were then adjusted to pH 5.0, and assayed on leaves of P. tremuloides clone 5. Isolates of H. mammatum varied in ability to produce toxin in culture (Figure 8).

To determine if toxin production by different isolates was related

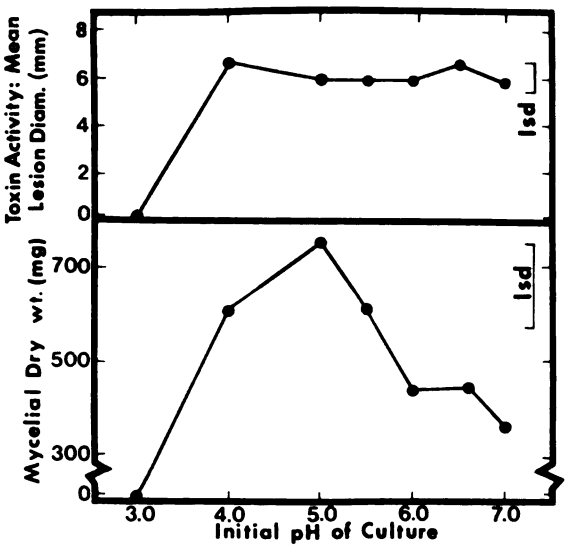

Figure 6. Toxin production (upper graph) and mycelial growth (lower graph) by H. mammatum. Cultures were initiated at pH 3.0 to 7.0, and maintained for 28 days. Each point in the lower graph represents the mean of 3 replicate cultures. Filtrates were then adjusted to pH 5.0, and assayed for toxicity on two replicate P. tremuloides leaves from two trees of clone 5 (upper graph). Least significant difference (lsd) at P <.05, among points greater than zero is indicated.

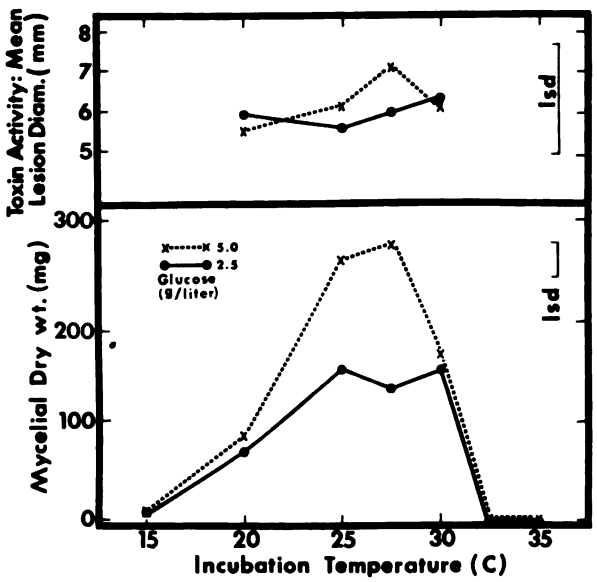

Figure 7. Toxin production (upper graph) and mycelial growth (lower graph) by H. mammatum in media containing 2.5 or 5.0 g/liter glucose. Cultures were incubated at 15-35 C for 21 days. Each point in the lower graph represents the mean of 3 replicate cultures. Replicate culture filtrates were pooled and assayed for toxicity on 4 replicate P. tremuloides leaves from 4 trees of clone 5 (upper graph). Least significant difference (lsd) at P <.05, among points greater than zero is indicated.

to their virulence, the same five isolates were inoculated into three-year-old main stems of ramets of P. tremuloides clones 2, 3, and 5, using previously described techniques (6, 7). Trees were 1 to 1.5 m tall, and were maintained in 14 cm pots in the greenhouse under continuous light (13 - 14 hr daylight, supplemented with 12 hr fluorescent light). Trees were watered daily, fertilized weekly with Plant Marvel (20-20-20, wt:wt:wt, of available N, P, and K, respectively) in water solution, and supplemented during the second week of the experiment with $MgPO_4$ in a soil drench. Each tree was inoculated 20 cm from the soil line with a single H. mammatum isolate growing on wheat grain. Six replications of each clone - isolate combination were used.

Cankers were measured 56 days after inoculation from apical to basal ends after removal of outer bark (5). Cankers developed as described in previous tests (5) from inoculations with isolates RL5-2, RL5-7, and 211-8, while isolates 605-6 and RL5-8 were low in virulence. Isolate 605-6 caused two small cankers on stems of trees in clone 5. Mean canker length produced by each isolate on clones 2, 3, and 5 was plotted against the mean diameter of lesions produced by culture filtrates of the same isolates when assayed on leaves of clones 2, 3, and 5 (Figure 9). Significant linear correlations were observed using stems and leaves of clones 3 and 5. Clone 2 was sensitive to toxin from only one isolate in leaf assays, but developed cankers from inoculations with all three isolates of H. mammatum with highest virulence.

Eighteen single spore isolates and three mass isolates of H. mammatum all produced toxin in culture. Eighteen of these isolates were pathogenic in previous inoculation tests (6, 7), and the remaining three isolates were generally non-pathogenic on most inoculated clones of P.

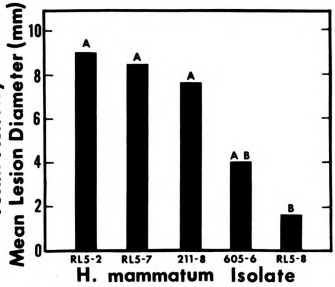

Figure 8. Toxin production by 5 isolates of Hypoxylon mammatum. Pooled filtrates from each isolate culture were adjusted to equal volume per mg of mycelial dry weight, and to pH 5.0, then assayed on 4 replicate leaves of P. tremuloides clone 5. Mean diameters surmounted by a common letter are not significantly different (P <.05).

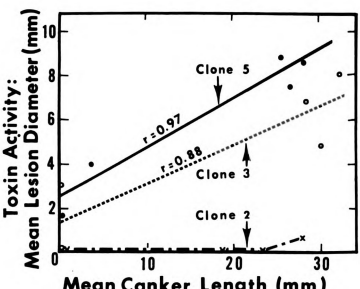

Figure 9. Toxin production vs. canker length caused by 5 isolates of H. mammatum. Each point represents toxicity of culture filtrates (4 replicate leaves) plotted against mean canker length (6 replicate stems) caused by each isolate when tested on P. tremuloides clones 2, 3, and 5. Regression lines for clones 3 and 5 were significant (P <.05).

tremuloides. Isolates differed, however, in size of lesions which were induced by their culture filtrates. Non-pathogenic isolates or those of low virulence produced the smallest amounts of toxin.

Variability of host response to toxin.- Lyophilized toxin was dissolved in water (8.0 mg/ml), diluted serially to 1.0 mg/ml, and assayed on leaves of P. tremuloides clones 1, 2, 3, 4, and 5. Leaves from clones 3 and 5 were highly sensitive to toxin, and produced significantly larger lesions than leaves from clones 1, 2, and 4 (Figure 10 and Table 3).

Leaves from 24 additional clones were tested with concentrated, deproteinized culture filtrate of isolate RL5-2. Again, significant differences in toxin sensitivity among clones were observed (Table 4).

To determine if varying sensitivity to toxin among clones was related to their susceptibility to H. mammatum, four three-year-old stems of P. tremuloides clones 1, 2, 3, 4, and 5, and six one-year-old stems of each of 24 clones (represented in Table 4), were inoculated with H. mammatum isolate RL5-2. The twenty three-year-old stems were inoculated in mid-March in the greenhouse at the time of leaf flush after a two-month period of dormancy in outdoor cold frames. Trees were maintained as above under continous light in 30 cm diameter pots. One-year-old stems were inoculated at the same time, but these plants had been held in a state of continous growth in the greenhouse since their propagation during the preceding summer. Stems were maintained in two-inch square Plant Band containers (Monarch Mfg. Co., Salida, Colo.), under the same lighting, watering, and fertilization regimes described above. Cankers developed on 162 of 163 inoculated stems. One-year-old stems were harvested 24 days after inoculation, three-year-old stems 56 days after inoculation. Cankers were examined in the laboratory under 7x

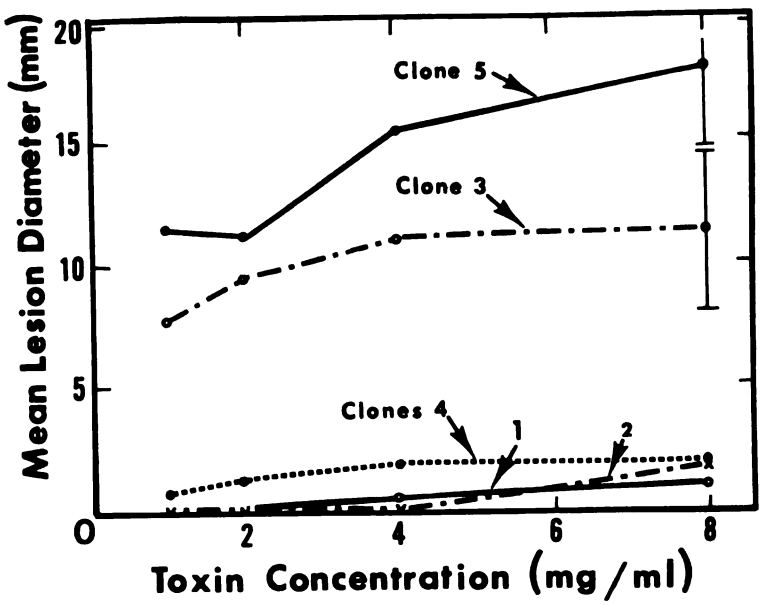

Figure 10. Sensitivity of 5 Populus tremuloides clones to lyophilized H. mammatum toxin. Each point represents the mean diameter of lesions on 4 replicate leaves of each clone. Confidence intervals (95%) around points indicating exposure of clones 3 and 5 to 8 mg/ml toxin are delineated.

Table 3. Response of 5 genotypes of Populus tremuloides to inoculations with Hypoxylon mammatum isolate RL5-2, and to its host-selective toxin. Means in each column followed by a common letter are not significantly different (P <.05).

Clone	Mean Lesion Diameter (mm)		Mean Canker Length (mm)	
5	18.0 [a/]	A	35.0 [b/]	Z
3	11.3	B	66.0	X
4	1.8	C	60.0	XY
2	1.8	C	32.8	Z
1	0.9	C	39.8	YZ

a/ Mean diameter of lesions caused by exposure of 4 replicate leaves to 8.0 mg/ml lyophilized toxin (see Fig. 10).

b/ Mean length of cankers among 4 replicate three-year-old stems.

Table 4. Response of 24 genotypes of Populus tremuloides to inoculations with Hypoxylon mammatum isolate RL5-2, and to its host-selective toxin. Means in each column followed by a common letter are not significantly different (P <.01).

Clone	Mean Lesion Diameter (mm)		Mean Canker Length (mm)	
281	20.8[a/]	A	41.2[b/]	VWXYZ
201	19.5	A	39.8	VWXYZ
71	15.5	A	52.2	UVW
242	9.4	B	37.0	XYZ
32	9.1	B	31.5	WXYZ
62	8.0	BC	19.0	Z
203	7.9	BC	71.3	U
404	6.8	BCD	32.8	WXYZ
64	6.0	BCDE	39.5	WXYZ
97	5.9	BCDE	51.3	UVWX
94	5.4	BCDEF	34.2	WXYZ
243	5.0	BCDEF	33.2	WXYZ
34	4.9	BCDEF	56.4	UVW
9	4.9	BCDEF	47.5	UVWXY
8	4.6	CDEF	41.3	VWXYZ
63	4.4	CDEFG	37.5	WXYZ
66	3.3	DEFG	31.8	WXYZ
33	2.8	DEFG	66.0	UV
6	2.8	DEFG	21.5	YZ
74	2.3	DEFG	37.7	WXYZ
69	2.3	DEFG	22.8	YZ
405	1.4	EFG	42.2	VWXYZ
406	1.1	FG	42.0	VWXYZ
412	0.0	G	25.7	XYZ

a/ Mean diameter of 8 lesions caused by exposure of 2 replicate leaves to concentrated, deproteinized culture filtrate.

b/ Mean length of cankers among 6 replicate one-year-old stems.

magnification, and measured from apical to basal ends after removal of outer bark.

Canker lengths varied significantly among clones of both three-year-old and one-year-old stems (Tables 3 and 4). Mean canker length observed on each clone was plotted against sensitivity to toxin in assays of leaves from the same clone. Among the three-year-old stems of clones 1, 2, 3, 4, and 5, mean canker length was plotted against the mean diameter of lesions on leaves exposed to 8.0 mg lyophilized toxin per ml (Table 3, from Figure 10). No significant correlation between sensitivity to toxin and size of cankers was observed among these five clones. Among the one-year-old stems of 24 P. tremuloides clones, mean canker length was plotted against the mean diameter of lesions on leaves exposed to concentrated, deproteinized culture filtrate of isolate RL5-2 (Table 4). Again, no significant correlation between lesion size and size of cankers was observed.

The 24 clones were then statistically grouped according to their toxin sensitivity. For example, clones 281, 201, and 71, which were not significantly different in their sensitivity to toxin, comprised group A. Group G included clones 63 through 412 (see Table 4). The mean toxin sensitivity of all clones in each group was then calculated, as well as the mean length of all cankers on all clones in each group (Table 5). Linear regression analysis between the two values thus obtained for groups A through G, indicated a significant, positive relationship between toxin sensitivity and mean canker length ($r = 0.87$).

Table 5. Response of 24 clones of Populus tremuloides to inoculations with Hypoxylon mammatum isolate RL5-2, and to its host-selective toxin. Clones are ranked in groups (A through G) not significantly different in toxin sensitivity (P <.01).

Clone Group	Mean Lesion Diameter (mm)	Mean Canker Length (mm)
A	18.60 a/	44.40 b/
B	6.66	41.25
C	6.08	41.29
D	4.39	39.54
E	4.00	40.21
F	3.48	39.55
G	2.27	36.36
	r = 0.87 c/	

a/ Mean diameter of lesions caused by exposure of 2 replicate leaves of all clones in each group to concentrated, deproteinized filtrate.

b/ Mean length of cankers among 6 replicate one-year-old stems of all clones in each group.

c/ Correlation coefficient (r) between the two indices is significant at 1% error probability.

DISCUSSION

These experiments corroborate earlier evidence (15, 16) of production of host-selective metabolites by H. mammatum in culture. Such compounds were regular metabolic products of the fungus, produced under all conditions tested that allowed growth of mycelium. Toxic metabolites were active only on certain Populus species, and produced spreading, black necrotic lesions only on leaves of P. tremuloides, which is the major host of H. mammatum.

The effectiveness of leaf assays in determining toxin concentration or activity was demonstrated by use of lyophilized, partially purified toxin supplied to leaves in various concentrations. The linear dosage response relationship between lesion size and toxin concentration (plotted logarithmically) indicates that lesion size is related to the concentration of toxin applied to the leaf. Leaves of P. deltoides, which is not infected with H. mammatum in nature, were not affected by toxin, either in the form of crude culture filtrate, concentrated, deproteinized culture filtrate, or when lyophilized toxin was supplied to the leaf. Schipper also reported low sensitivity of P. deltoides leaves to H. mammatum toxin (15).

Chromatography of toxic components on silica gel plates indicated that at least two toxic compounds may be produced by H. mammatum. This is in agreement with results obtained by Schipper (17) which indicated that up to four host-selective compounds could be extracted from rye grain cultures of the fungus. In the case of H. mammatum toxin(s) it

is not known whether all or indeed any of the compounds indicated herein are produced during actual infection of stems. However, host-selective compounds have been isolated from bark infected with H. mammatum (15).

The toxic components identified in these experiments are compounds of low molecular weight (less than 1000 d). Toxic activity of culture filtrates was not lost by removal of large compounds either by methanol precipitation of proteins and polysaccharides, or by their separation on dextran molecular sizing gels. Toxin was partitioned into n-butanol, leaving salts and yellow-colored compounds in the aqueous phase. These procedures could be useful for further purification and characterization of toxic compounds. Also, the thermal stability of toxic components may facilitate their characterization via gas chromatography and mass spectrometry.

Toxin production by different isolates of H. mammatum varied significantly. The ability to produce toxin by five isolates in culture was directly related to their ability to produce cankers when inoculated into stems of P. tremuloides clones 3 and 5. In tests of 21 isolates of H. mammatum, all isolates produced toxin, but the lowest amounts were produced by isolates which were essentially non-pathogenic [isolate 605-6 (6), and isolates RL5-4, RL5-8]. These three non-pathogenic isolates all demonstrated the 'conidial' growth form in vitro (2), which has been associated with low pathogenicity (2, 6). It seems, therefore, that a threshold level of toxin production may be necessary for infection of P. tremuloides stems. Previous evidence also indicated that presence of toxin was necessary for infection by H. mammatum (16).

Sensitivity to H. mammatum toxin(s) may be related to

susceptibility of aspens to infection by the fungus. Leaves from clones of P. grandidentata, which is rarely infected in nature, developed small, brown lesions after treatment with toxin. In contrast, large, black lesions were observed on P. tremuloides leaves. Toxin completely blackened the leaves of some clones after exposure of up to 48 hr. The significant variation among P. tremuloides clones in sensitivity to toxin in leaf assays might indicate their variation in susceptibility to H. mammatum. Attempts to prove this relationship by inoculating young stems in the greenhouse were largely unsuccessful, however. Sensitivity to toxin in leaf assays was generally not correlated with size of cankers produced by inoculation of stems with the fungus. Lack of correlation between the two values was attributed to clones such as 33 and 34 (Table 4), which produced long cankers after inoculation with H. mammatum, but were low in sensitivity to toxin. Alternatively, clone 63 was highly sensitive to toxin, but produced short cankers after inoculation.

When clones were grouped statistically according to their toxin sensitivity, however, a direct relationship of toxin sensitivity with canker length was indicated (Table 5). This type of grouping may compensate for spurious results which might be produced by any single clone in either toxin sensitivity or rate of canker development after inoculation. Also, inoculation of young main stems of P. tremuloides clones may not be an optimal procedure for determining susceptibility to Hypoxylon canker. Infection in nature is most commonly associated with lateral branches of sapling-sized trees following insect attack or branch death (10, 11). The relationship between toxin sensitivity and susceptibility of clones to infection by H. mammatum must be evaluated

more rigorously before attempting to use H. mammatum host-selective metabolites as a tool for screening for resistance.

At present, it is recommended that procedures for identifying resistance to Hypoxylon canker include both inoculation with the fungus and tests with its host-selective toxin. Genotypes which emerge as desirable in both respects could be used for establishing plantations. These could then be evaluated for any long-term gain in disease resistance after exposure to conditions of natural inoculation and infection.

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SUMMARY

Identifying aspen genetically resistant to Hypoxylon canker and establishing such stock in plantations may be valuable contributions toward increasing the production of raw materials for forest based industries. Losses to the disease could be reduced in this manner, allowing a greater portion of the aspen resource to be converted to usable cellulose.

Identification of resistance within Populus tremuloides may be accomplished through study of naturally existing clones, selecting genotypes either low in amounts of natural infection, or which produce short cankers after inoculation with Hypoxylon mammatum. The present experiments indicate that inoculation with the fungus may be generally applied over wide geographical areas, and that clone differences in canker length may be readily detected, provided that standardized inoculum types and techniques are used. Moreover, this technique may be useful in areas where natural incidence of Hypoxylon canker is low, and where susceptibility among the host population may not be expressed due to environmental factors.

Resistance may also be expressed among aspen genotypes by low sensitivity to host-specific metabolites produced by H. mammatum in culture. P. grandidentata is virtually immune to natural infection by H. mammatum, and leaves from this species exhibited low sensitivity to toxic substances from culture filtrates of the fungus. P. tremuloides

leaves exhibited wide variability in sensitivity to the same metabolites, indicating that resistance may be present within this species also.

Extreme caution must be exercised in the application of these techniques, however. It is recommended that procedures for selection of resistant genotypes include both inoculation with the fungus under standardized conditions, and tests with its host-specific toxin(s). Neither technique alone is a proven indicator of resistance to Hypoxylon canker.

The procedures outlined in the present experiments may be useful since they are cheap and easily accomplished. The alternatives, which would normally include establishment of a random sample of aspen genotypes in a common plantation, followed by long-term evaluation of disease incidence, would involve unnecessary expenditure of time and space on genotypes with little promise of desirable qualities. Preliminary screeing of P. tremuloides clones in situ by inoculation with fungus mycelium, or by use of excised leaves in assays of toxin sensitivity, would allow a proper focus on genotypes which are potentially desirable from both aspects. Such genotypes could then be used for plantation or for hybridization with P. grandidentata. Hybrids between these two species have demonstrated advantages in resistance to canker enlargement; use of higher levels of resistance in P. tremuloides parental lines may provide even greater gains in this respect.

APPENDIX

Appendix A. Mean canker lengths caused by inoculation of four clones of *Populus tremuloides* with 12 isolates of *Hypoxylon mammatum*, during June, July, and August, 1974.

	June							
Clone	RL 11		RL 1		RL 8		RL 4	
Isolate	no. cankers	mean length	no. cankers	mean length	no. cankers	mean length	no. cankers	mean length
RL5-2(A)	4	92	3	64	3	71	3	60
RL5-6(B)	4	72	4	73	3	61	4	44
RL5-3(C)	4	84	3	62	3	51	3	55
RL5-7(D)	4	75	4	53	4	46	4	46
211-2(E)	2	99	4	65	3	82	2	74
211-8(F)	3	56	4	34	4	43	2	57
RL5-5	4	46	0	-	1	47	2	50
208-6	4	77	0	-	0	-	0	-
RL5-8	0	-	1	21	0	-	0	-
RL5-1	1	16	0	-	0	-	0	-
605-6	0	-	0	-	0	-	0	-
RL5-4	0	-	0	-	0	-	0	-
control	0	-	0	-	0	-	0	-
All Isolates	30	72	23	56	21	57	20	51
All Clones	no. cankers = 94 mean canker length = 60.8							

Appendix A, continued

	July							
Clone	RL 11		RL 1		RL 8		RL 4	
Isolate	no. cankers	mean length	no. cankers	mean length	no. cankers	mean length	no. cankers	mean length
RL5-2(A)	3	80	4	47	3	52	3	38
RL5-6(B)	4	62	4	63	4	52	3	36
RL5-3(C)	2	61	4	60	3	45	4	41
RL5-7(D)	4	64	4	58	2	42	1	18
211-2(E)	2	78	4	82	4	71	2	30
211-8(F)	4	61	4	35	4	38	3	24
RL5-5	1	44	2	29	0	-	3	32
208-6	0	-	0	-	0	-	0	-
RL5-8	0	-	0	-	0	-	0	-
RL5-1	0	-	0	-	0	-	0	-
605-6	0	-	0	-	0	-	1	21
RL5-4	0	-	0	-	0	-	0	-
control	0	-	0	-	0	-	0	-
All Isolates	20	65	26	55	20	51	20	33
All Clones	no. cankers = 86 mean canker length = 51.3							

Appendix A, continued

	August							
Clone	RL 11		RL 1		RL 8		RL 4	
Isolate	no. cankers	mean length	no. cankers	mean length	no. cankers	mean length	no. cankers	mean length
RL5-2(A)	4	37	4	14	4	14	3	28
RL5-6(B)	3	23	4	24	4	21	3	16
RL5-3(C)	2	23	4	18	4	19	4	15
RL5-7(D)	1	35	0	-	2	21	2	13
211-2(E)	0	-	0	-	2	32	0	-
211-8(F)	0	-	0	-	0	-	0	-
RL5-5	0	-	0	-	0	-	0	-
208-6	0	-	0	-	0	-	0	-
RL5-8	2	12	0	-	1	12	0	-
RL5-1	0	-	0	-	0	-	0	-
605-6	0	-	0	-	0	-	0	-
RL5-4	0	-	0	-	0	-	0	-
control	0	-	0	-	0	-	0	-
All Isolates	12	27	12	18	17	20	12	18
All Clones	no. cankers = 53 mean canker length = 20.6							

Appendix A, continued

Clone / Isolate	All Months								All Clones	
	RL 11		RL 1		RL 8		RL 4			
	no. cankers	mean length	no. cankers	mean length	no. cankers	mean length	no. cankers	mean length	no. cankers	mean length
RL5-2(A)	11	69	11	40	10	43	9	42	41	48.6
RL5-6(B)	11	55	12	53	11	44	10	33	44	46.6
RL5-3(C)	8	63	11	45	10	36	11	35	40	43.7
RL5-7(D)	9	66	8	55	8	38	7	32	32	48.9
211-2(E)	4	88	8	74	9	66	4	52	25	69.6
211-8(F)	7	59	8	35	8	40	5	37	28	42.7
RL5-5	5	46	2	29	1	47	5	39	13	40.8
208-6	4	77	0	-	0	-	0	-	4	76.5
RL5-8	2	12	1	21	1	12	0	-	4	14.3
RL5-1	1	16	0	-	0	-	0	-	1	16.0
605-6	0	-	0	-	0	-	1	21	1	21.0
RL5-4	0	-	0	-	0	-	0	-	0	-
control	0	-	0	-	0	-	0	-	0	-
All Isolates	62	61	61	48	58	44	52	37	233	48.2

Appendix B. Mean canker lengths (MCL) caused by inoculation of 100 clones of Populus tremuloides with two highly virulent isolates of Hypoxylon mammatum, during 1975.

area	clone	no. cankers	MCL RL5-2	MCL RL5-6	MCL RL5-6 + MCL RL5-2	combined sum of MCL all clones, each area	natural infection: no. trees tallied	natural infection: % infected
1	1	5	67	55	123		21	33
	2	5	72	50	123	a/	28	43
	3	5	64	54	118	AB	31	23
	4	4	53	45	98	105.5	29	28
	5	4	51	39	90		21	38
	6	5	47	31	78		22	0
2	1	5	73	52	126		38	3
	2	5	67	56	123		26	58
	3	5	73	43	116	AB	20	15
	4	5	60	42	102	101.8	41	2
	5	5	60	35	96		23	0
	6	5	48	47	95		24	17
	7	5	55	36	91		20	42
	8	5	46	21	67		66	0
3	1	5	78	51	129		20	10
	2	5	71	57	129		20	15
	3	5	66	57	123		38	26
	4	5	71	51	122		35	40
	5	5	73	48	122	AB	23	0
	6	5	77	44	121	117.0	34	24
	7	5	57	53	109		33	18
	8	5	59	45	105		65	14
	9	4	59	46	104		20	6
	10	2	48	37	85		20	0
4	1	4	79	58	138		20	6
	2	4	73	47	120		79	9
	3	5	63	48	111		41	7
	4	5	56	52	108	AB	45	29
	5	4	62	42	104	110.5	35	0
	6	5	63	41	104		40	23
	7	4	51	40	92		64	11
5	1	5	81	74	155		43	5
	2	4	78	69	148		54	14
	3	5	61	62	123		42	12
	4	5	66	54	120	AB	51	20
	5	5	65	51	116	118.1	81	6
	6	5	61	42	103		62	10
	7	4	47	47	94		75	36
	8	5	56	32	87		74	8

Appendix B, continued

6	1	5	82	60	142		85	53
	2	5	80	61	141		66	5
	3	5	63	66	130		27	33
	4	4	69	47	116		24	38
	5	5	55	50	104	AB	57	40
	6	4	50	51	102	113.0	42	10
	7	5	68	32	100		27	11
	8	5	63	35	98		51	35
	9	5	57	38	95		55	4
	10	2	33	49	82		78	0
7	1	3	66	77	143		47	19
	2	5	71	70	140		30	13
	3	3	72	55	127		35	43
	4	3	54	56	110		94	39
	5	4	51	50	101	AB	39	31
	6	2	52	46	98	113.8	50	22
	7	4	46	50	96		41	7
	8	2	49	43	92		53	2
	9	2	41	48	88		62	10
8	1	4	78	74	151		60	27
	2	5	81	61	141		52	12
	3	5	73	61	134		35	23
	4	5	65	67	132	A	41	10
	5	5	75	52	127	124.9	59	7
	6	5	68	47	115		22	0
	7	5	66	45	111		53	11
	8	5	52	41	93		66	21
9	1	5	96	59	115		100	1
	2	4	68	65	133		10	0
	3	5	74	52	125	AB	33	0
	4	5	66	46	113	122.1	71	1
	5	5	59	48	108		53	0
	6	5	56	45	101		20	0
10	1	5	66	53	119		100	7
	2	5	64	47	110		38	8
	3	4	62	44	105		26	4
	4	5	54	46	100		38	9
	5	5	56	40	97	AB	42	5
	6	5	53	39	92	95.4	43	7
	7	4	49	42	91		30	23
	8	5	49	39	88		25	4
	9	5	37	40	77		20	0
	10	5	50	27	76		29	17

Appendix B, continued

11	1	3	51	43	94		48	33
	2	4	36	33	69		25	21
	3	1	44	24	68		32	19
	4	5	31	34	65		34	44
	5	4	34	29	63	B	41	10
	6	5	35	27	62	63.6	83	11
	7	5	38	23	62		52	21
	8	4	33	28	61		50	16
	9	5	32	26	58		78	23
	10	3	23	20	43		63	4
12	1	5	52	44	95		32	41
	2	2	53	40	93		25	8
	3	5	43	35	77		52	44
	4	5	44	31	75	B	100	38
	5	5	38	26	64	68.7	74	20
	6	5	32	26	57		75	45
	7	4	24	29	53		48	10
	8	4	25	15	40		20	0

a/ Among areas, combined sums of mean canker length surmounted by a common letter are not significantly different ($P < .05$).