Host-Pathogen Interactions as Measured by Bioassay of Metabolites as Produced by *Hypoxylon mammatum* with its Host *Populus tremuloides*

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ABSTRACT

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Five single-ascospore isolates of *Hypoxylon mammatum* were used to produce metabolites in a chemically defined broth medium and in grain cultures. After treatment to remove high-molecular-weight materials, the culture filtrates and grain culture extracts were concentrated and tested for toxic activity against nine clones of *Populus tremuloides* by using a leaf-puncture bioassay. The intensity of the reaction was measured by testing serial dilutions of the samples, and sensitivity of the host was measured according to the size of lesion produced at each dilution. The response was dependent on time and concentration of the materials. Both metabolite production methods gave preparations that yielded similar results. The aspen clones could be classified into three sensitivity groups by the bioassays, high, medium, and low. Analysis of variance indicated that horizontal response (clone main effects) was most important, but significant (although quantitatively less important) vertical response mechanisms (clone by isolate interactions) were also present. The host reactions were also compared to canker development data and showed negative correlation to canker length and positive correlation to frequency of callus formation. These correlations suggest that the bioassay of the sensitivity to the metabolites produced in culture by these methods measures the ability of the aspen clones to respond to elicitors. Metabolite-producing abilities of the fungal isolates were not related to their canker-forming abilities when inoculated into the trees.

Hypoxylon mammatum (Wahl.) Miller causes a stem canker in aspen (*Populus tremuloides* Michx.), and probably is the most significant pathogen of this species (3,16). Studies of host-pathogen interactions in stem canker have included studies on canker development (1,2,10–12,14) and the effects of toxic metabolites produced in culture (1,5,9,13,15,19,20). These studies have given valuable insights into the pathogenicity of the fungus and corresponding resistance mechanisms in the host that could be selected in a breeding program for improving the crop potential of aspen.

The use of host-selective metabolites of the fungus for a rapid bioassay of host resistance mechanisms could be a valuable tool in a selection program, but it requires an understanding of the role of metabolites in the disease process and of the ways in which host response to metabolites is related to resistance mechanisms. There have been several attempts to isolate and identify the toxic metabolites produced by *H. mammatum* (10,13,19,20). Although these investigations have yielded much information on the properties of these compounds, none have reported purification or identification of them.

Circumstantial evidence that the metabolites produced in pure culture play a role in canker development has been obtained (1,9,10,19,20). However, several reports indicate that the relationship of sensitivity to these metabolites to disease susceptibility may be complex. For example, there was no correlation between canker length produced in field inoculations and sensitivity to toxins (10). Fungal isolates that produced cankers in experimental inoculations did not always produce metabolites in culture that were active against the same aspen clones (10,19,20). The high correlation of disease incidence in wild clones with sensitivity to toxic culture filtrates produced by a single fungal isolate (5) was not reproducible in a subsequent investigation of the same clones but different fungal isolates (9). The complexity of

possible relationships of metabolites to the disease process was suggested by the complexity in the numbers of toxic metabolites produced (10,13,19,20).

Some of the inconsistencies in these reports could be explained by environmental effects (4,11). Some inoculation studies involved wild aspen clones where site factors could have had significant effects on canker development (10–12). Another possible source of variation is the culture media for metabolite production which have included rye grain (19), malt agar (1,15), a yeast extract medium (20), and chemically defined media (5,9,13).

In the present study, we determined the general responses of the aspen clones to a mixture of toxic metabolites, since they will be challenged by a mixture in the canker infection. We examined two very different production media, rye grain solid culture and a chemically defined liquid medium with submerged culture, to determine whether differences in responses occur that might explain the discrepancies that have been reported. We compared these responses to other measures of canker susceptibility to provide a better understanding of how the metabolite bioassay might be used in a selection program for canker-resistant aspen. The measures of susceptibility used for these comparisons were natural incidence of infection in the wild clones at their original site of collection, natural incidence of infection in the same clones after 10 yr of growth in a garden experiment, and canker development measurements from experimental inoculations of the same clones in the garden situation where site factors were controlled (14).

MATERIALS AND METHODS

The isolates of *H. mammatum* and aspen clones used in these experiments were described previously (14). The five fungal isolates and five of the aspen clones were also tested by French and Manion (12). The fungal isolates can be related to the latter study by their numeric designation (208-6, 209-5, 209-6, 605-1, and 608-7). The first three digits refer to the aspen clone serving as the inoculum source, and the final digit refers to the ascospore number (Table 1 of reference 12). All of these isolates were mycelial-type isolates and grew vigorously. The clones are referred to by the same numbers in both studies.

To produce the culture filtrates in a defined medium, the isolates were first grown on an agar medium (1.25 g of peptone, 1.25 g of yeast extract, 5 g of glucose, and 15 g of agar per liter) for 6–14 days at 28 C. Twenty mycelial plugs of each isolate were taken from the growing margins of the colonies with an 8-mm-diameter cork borer and macerated in 10 ml of sterile deionized water in a Sorvall Omnimixer. This slurry was transferred to three 250-ml Erlenmeyer flasks (2.5 ml of slurry per flask) containing 50 ml of a defined medium (9). The flasks were incubated at 28 C on a rotary shaker with a 2.5-cm amplitude at 100 rpm for 10 days. The mycelia from three flasks were filtered together, washed with sterile deionized water, and macerated in 20 ml of sterile water. The entire macerate was added to 2.4 L of defined medium, which was dispensed 800 ml per 1.5-L Erlenmeyer flask, and incubated on the rotary shaker as before. After 10 days, the mycelium was removed by filtration, and the combined filtrates of three flasks were concentrated to about 500 ml on a flash evaporator. An equal volume of cold methanol was added and filtrates were stored overnight at −15 C. The precipitate was removed by filtration and the filtrates were taken to dryness in a flash evaporator. The residues were dissolved in 70% methanol equal to 1% of the initial volume of culture filtrates. Rye grain cultures (19) were inoculated with mycelial macerate produced as described above and incubated at 28 C for 14 and 21 days. Metabolites were extracted as described by Schipper (19).

Concentrated culture filtrates in 70% methanol were diluted 1-, 4-, 16-, 64-, 256-, and 1,024-fold with 70% methanol for a leaf bioassay test. For regression analysis, this logarithmic series was transformed to a dilution number equal to the $\log_4$ of the dilution, i.e., 0, 1, 2, 3, 4, and 5, respectively. The bioassay test was similar to that described by Bruck and Manion (5) except that 2-μl drops of test material were applied to the leaf-puncture wounds. Lesion diameters were measured at 24, 48, and 72 hr. Leaves for the bioassays were obtained from ramets of nine clones grown from root sprouts maintained in a greenhouse with supplemental lighting to maintain 16-hr day lengths. The leaves were removed by cutting the petioles flush with the stem and immediately placing the petioles in tubes containing deionized water. A total of five complete dilution series were tested for each isolate's metabolite preparation on each clone. For the metabolites from grain cultures, four separate cultures were grown for each isolate. The metabolites were extracted separately from each culture and a single dilution series from each culture was tested on each clone for a total of four replicated tests for each isolate on each clone.

The disease incidence data in the wild clones were collected in 1970 by counting the number of diseased and healthy trees in a 1.8-m transect through the center of each clone. Only trees 5 cm or greater at 1.75 m from the ground were included. The disease incidence data in the garden (14) was collected by examining each of the 27 ramets planted in the garden for the presence of the disease.

Statistical analysis was by the computer program package provided by the SAS Institute, release 82.3B (17), at Syracuse University. Comparisons of the simultaneous regression functions of the various clones were made by using the used dummy variable techniques described by Cunia (6) and Draper and Smith (7).

RESULTS

The leaf lesions produced by these culture filtrate preparations were black, similar to those reported by French (10) and Schipper (19), and unlike the tan lesions with black rings reported by Bruck and Manion (5). The isolate used by Bruck and Manion (5) has not survived, but a related isolate from the same source also produced black-ringed tan lesions; these are the only two isolates of the 16 that we have examined that produce this unique reaction. Furthermore, the concentrated extracts of the surviving isolate that produced the brown reaction were very acidic (less than pH 3). On neutralization to pH 3 or higher, the rapidly appearing tan reaction no longer occurred, and the relatively slow-developing black lesion reaction was observed. Strong acids at pH 3 or below (10^{-2}–10^{-3} M) also gave the black-ringed tan lesions; these included phosphoric acid, sulfuric acid, and citric acid. Hydrochloric acid and acetic acid gave black lesions. The preparations used in this study were adjusted to pH 7 with KOH.

The black lesions appeared in 12–72 hr depending on the concentration, clone, and isolate. After a brief lag in reaction, the
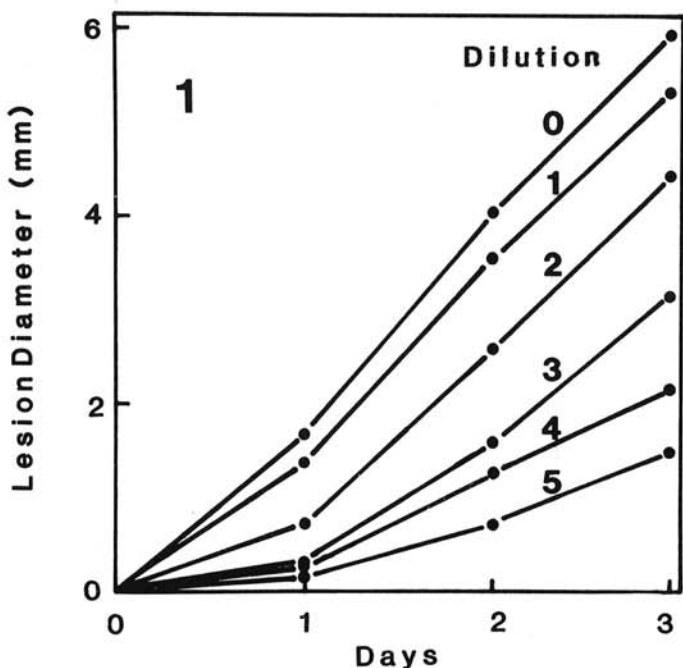
Fig. 1. Effect of time and dilution on the mean leaf lesion diameters for all tests of *Hypoxylon mammatum* on *Populus tremuloides*. Dilution is expressed as $\log_4$ of the original concentrated extract.
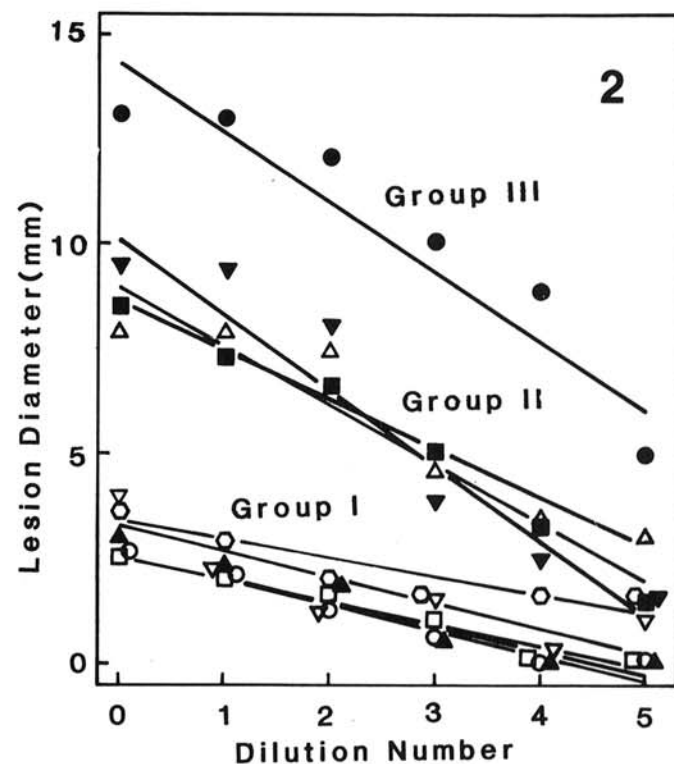
Fig. 2. The effect of dilution of extracts from cultures of *Hypoxylon mammatum* in a defined medium on the mean lesion diameters of each aspen clone. Symbols for clones: 208 (o), 209 (□), 210 (•), 211 (■), 604 (▽), 608 (◯), 609 (▲), 610 (▼), and 611 (△). Isolates of *H. mammatum* have membership in Groups I, II, and III as shown in Table 1.

lesions increased linearly in size from day 1 to day 3 (Fig. 1). Observations beyond 3 days were not practicable because of confluence of the larger lesions and deterioration of the leaves. The data in Fig. 1 show that lesion size was dependent both on time and concentration of the metabolites. Uninoculated medium controls

for the submerged cultures and the rye grain cultures prepared in the same way as the experimental material gave no reaction in the bioassay.

The mean lesion diameters on leaves of the clones for all isolates to the two sets of metabolites are shown in Figs. 2 and 3. The simultaneous regression technique (6,7) was used to classify the clones into three groups with similar slopes and intercepts. This is a much more powerful technique for distinguishing differences between clones than analysis of variance with a multiple range test, because it uses data from the entire dilution curve rather than the response data from only one metabolite concentration at a time. The intercept and slope parameters were calculated by least-squares regression for each of these regressions with the equation $D = \beta_1 + \beta_2 T$ in which $D =$ lesion diameter, $T =$ metabolite dilution number, and β_1 and β_2 are the intercept and slope parameters, respectively. Analysis of the regressions within groups with the simultaneous regression method showed that the slopes and intercepts were not significantly different ($P \leqslant 0.01$). Between-group variation was significantly different except that group II and III slopes did not differ for the responses to metabolites from defined medium broth. The response curves of the clones to the two sets of metabolites were very similar.

The mean lesion diameters produced by the metabolite preparations of each isolate are shown in Figs. 4 and 5. The isolates may be classified into two groups with significantly different slopes only for the responses to the metabolites from grain cultures (Fig. 5). The slopes are not significantly different ($P \leqslant 0.01$) for the metabolites from defined medium. Even so, the response patterns of the isolates are remarkably similar for the two methods of metabolite production.

These regressions (Figs. 2–5) represent the means of each aspen clone for the metabolite preparations of all isolates (Figs. 2 and 3) and the means of each isolate's metabolite preparation tested on all clones (Figs. 4 and 5). These means measure the general response levels, but obscure the interactions between specific clones and isolates. Table 1 shows the intercepts for each clone by isolate combination with the metabolites produced in defined medium. Notice that Group I clones were most sensitive to metabolites from isolate 208-6, while Group II and III clones were most sensitive to metabolites from isolate 209-5 and least sensitive to those from isolate 208-6. Other differences can also be seen between specific clone by isolate combinations.
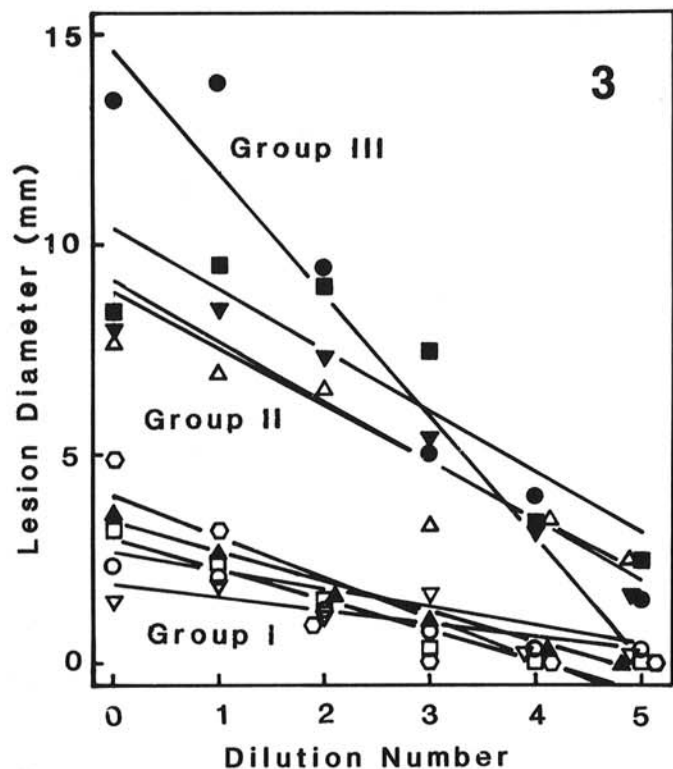

Fig. 3. The effect of dilution of extracts from cultures of *Hypoxylon mammatum* on rye grain on the mean lesion diameters of each aspen clone. Symbols for clones: 208 (o), 209 (□), 210 (●), 211 (■), 604 (▽), 608 (◇), 609 (▲), 610 (▼), and 611 (△). Isolates of *H. mammatum* have membership in Groups I, II, and III as shown in Table 1.

Fig. 4. The effect of dilution on the mean lesion diameters caused by the concentrates from the defined-medium cultures for each isolate of *Hypoxylon mammatum*. Symbols for isolates: 208-6 (●), 209-5 (□), 209-6 (△), 605-1 (■), and 608-7 (o).
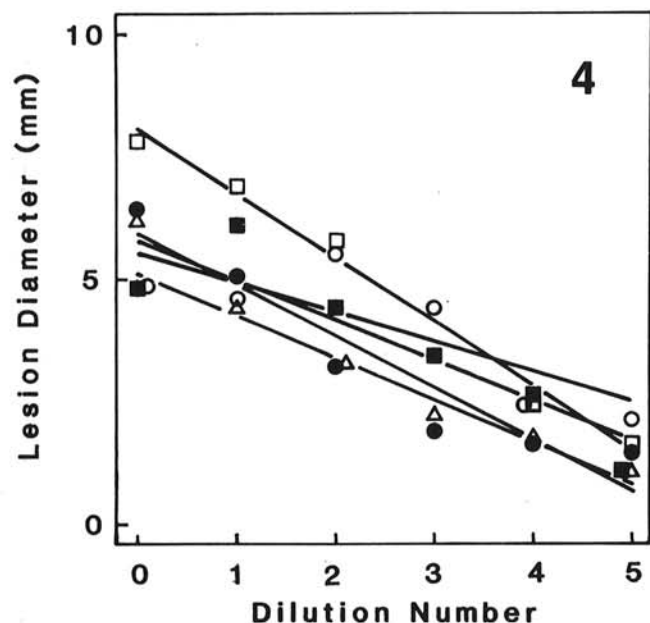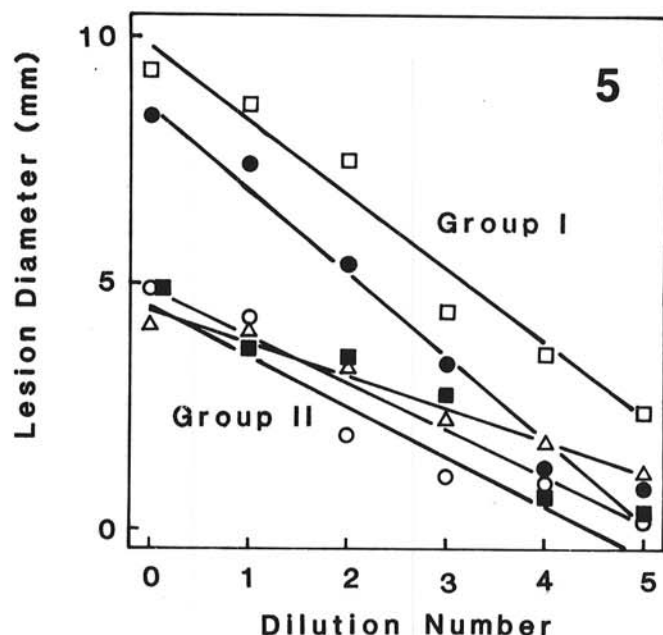

Fig. 5. The effect of dilution on the mean lesion diameters caused by the concentrates extracted from the grain cultures of each isolate of *Hypoxylon mammatum*. Symbols for isolates: 208-6 (●), 209-5 (□), 209-6 (△), 605-1 (■), and 608-7 (o).

The disease incidence at the collection site in the wild and in the garden plantings are shown in Table 2. There is no significant correlation between these two sets of data ($r = 0.26$, $P = 0.49$).

DISCUSSION

According to concepts of Vanderplank (21) on the use of analysis of variance, the clone main effect represents the measurement of horizontal resistance, the isolate main effect represents the measurement of pathogen aggressiveness (also a horizontal component) and the clone by isolate interactions represent measurement of the vertical resistance component. The analysis of variance for the measurements from the three highest concentrations of metabolites shows that horizontal component (r^2 for clones) accounts for the largest amount of variation, more than three times that of vertical component (r^2 for the interaction) (Table 3). Variation in fungal aggressiveness in the metabolite-producing capabilities of these isolates was significant, but accounted for only 3–4% of the total variation. This variation in horizontal resistance among the clones as described by the simultaneous regressions is the most important aspect of these tests.

The intercepts for the regressions of the clone by isolate combinations (Table 1) indicate a continuous range of variation in sensitivity to the various metabolite preparations, but this is not carried over to the clonal variation. The clones can be grouped into three distinct sensitivity groups, high, medium, and low. This type of grouping can also be seen in the results of French (10) and of Stermer et al (20). This small number of discrete response levels seen in the means of clones across all isolates and the largely non-overlap among these groups when considering the clone by isolate interactions, suggests that the primary effect of the interactions is to modify a basic response level determined by horizontally effective genes. The fact that only three horizontal response levels were obtained suggests that a small number of genes govern this response. This is contrary to the assumption that horizontal resistance is governed by a large number of genes with small additive effects (21).

The difference between the tan lesion symptoms reported by Bruck and Manion (5) and the black lesions reported by French (10), Griffin et al (13), and also in this study may be explainable as a pH phenomenon. Another major difference between the reactions in our study and those reported by Bruck and Manion (5) is the correlation of the response to natural disease incidence in the wild clones. Bruck and Manion (5) report a high positive correlation of disease incidence and lesion diameter in the wild clones ($r = 0.922$), whereas our results yield negative correlations ranging from $r = -0.58$ to -0.67 for the several dilutions of metabolites from defined medium. The high positive correlation of Bruck and Manion (5) involves a sample of only four clones. Ehrenshaft (9) tested the same four clones with different fungal isolates. The correlation

between disease incidence and metabolite response calculated from her data is -0.52, which is quite similar to our results.

In addition to disease incidence data from the wild clones, we have disease incidence from natural infections in the garden location where site factors are controlled and did not cause major response variations (14). The lack of correlation between the two sets of disease data could be the result of elimination of site factors in the clonal responses and to the relatively young age of the clones. Ten years in the field may not be sufficient time for full expression of the disease potential in these clones on the garden site. There were no significant correlations between disease incidence in the garden and the results of the metabolite bioassay, which could be indicative of the latter situation.

Since the identities of these compounds are not known we cannot be certain that the metabolites tested here were the same as those produced on infection of the aspen. However, the great similarity in response to the metabolites produced in submerged culture on defined medium and on the grain cultures suggests that similar compounds were produced and that the different growth conditions did not have a strong influence on their production. Alternatively, these results could mean that we have measured a general sensitivity that is not selective as to the specific metabolite (i.e., a horizontal response).

A comparison of the metabolite response data to the canker development data of the companion study (14) shows some interesting relationships between these responses. Sets of metabolites (from defined medium and from grain cultures) showed similar correlations with the canker development data. Canker lengths were negatively correlated with metabolite lesion diameters, with the highest levels of correlation occurring for the

TABLE 2. Disease incidence in the clones as surveyed on a transect through the clones in the wild and as surveyed in the garden planting

Clone no.	Disease incidence (%)	
	Wild[a]	Garden
208	42	24
209	33	4
210	16	8
211	11	11
604	20	15
608	16	11
609	44	15
610	17	20
611	20	17

[a]Correlation between Wild and Garden incidence is $r = 0.26$ ($P = 0.49$).

TABLE 3. Quantification of horizontal and vertical resistance responses of clones of *Populus tremuloides* and the pathogenic aggressiveness of *Hypoxylon mammatum* by analysis of variance of the metabolite sensitivity measurements for the three highest concentrations of toxins from the defined medium cultures

	Dilution number[a]					
	0		1		2	
Source	Prob.[b]	r^2[c]	Prob.	r^2	Prob.	r^2
Clone (horizontal response)	0.0001	0.37	0.0001	0.42	0.0001	0.40
Isolate (aggressiveness)	0.006	0.04	0.025	0.03	0.008	0.03
Clone × isolate (vertical response)	0.063	0.12	0.011	0.13	0.041	0.12
Replicates	0.014	0.03	0.12	0.02	0.48	<0.01
Total model	0.0001	0.56[c]	0.0001	0.60[c]	0.0001	0.60[d]

[a]Dilution number is $\log_4$ of the dilution series of 1-, 4-, and 16-fold dilutions, respectively.
[b]Prob. is the significance probability for the F test for each indicated source of variation.
[c]r^2 is the squared semipartial correlation (8) for each source of variation.
[d]For the total model, this is the coefficient of determination which is usually symbolized by R^2.

TABLE 1. Intercepts for the lesion diameter versus metabolite dilution regressions for each clone by isolate combination in tests of the metabolites produced by *Hypoxylon mammatum* in a defined medium

Group and clone	Isolates				
	208-6	209-5	209-6	605-1	608-7
Group I					
208	3.5	2.2	3.0	2.2	1.4
209	4.7	2.2	2.8	1.7	1.2
604	3.8	4.8	4.1	1.4	1.2
608	5.7[a]	1.6	2.4	1.6	5.0
609	4.6	3.6	1.9	1.9	1.4
Group II					
211	7.4	9.6	10.0	7.5	9.7
610	10.0	14.9	6.0	9.3	10.4
611	6.7	12.7	5.7[a]	9.8	8.0
Group III					
210	6.9	20.9	14.8	17.3	11.0

[a]These two clone by isolate combinations are the only overlapping intercepts between Group I and Group II clones.

canker lengths at 12 mo (−0.42 to −0.74 for grain culture metabolites and −0.77 to −0.83 for defined medium metabolites). Callus frequencies showed negative correlations to the metabolite bioassays at 4 mo, but the callus frequencies were very low at this time and significant differences between clones could not be demonstrated (14). At 12 and 16 mo, when callus frequencies were more fully developed, they showed positive correlations to the metabolite bioassay (0.60–0.83 for the highest metabolite concentrations). There were no consistent or significant correlations between branch death frequencies and the metabolite bioassays.

The positive correlation of metabolite bioassay response with callus formation response in the inoculations is the opposite of what would be expected for metabolites that are reported to inhibit callus formation when applied directly to stem wounds (19). The leaf bioassay may not reflect this stem response, but may be responding to other elements of the toxic culture filtrates. The response is correlated with the ability of the aspen to respond to the fungus by producing a resistance reaction (development of callus and limitation of canker elongation), and is not a measuring reaction to toxins per se. Although these metabolites are host selective (9,10,19), they do not appear to fit the same model as host-specific metabolites of fungi for annual crops (18) in which there is a direct relationship between metabolite production by the fungus, metabolite sensitivity of the host, and a compatible pathogenic interaction.

The disease model that is expressed with the hypoxylon stem canker is one of continuous variation from greater susceptibility to greater resistance, rather than the discontinuous model expressed in many agricultural crops (21). This continuous variation is seen in all measurements of susceptibility, disease incidence, canker length, callus response, and branch death (14). We see a consistent relationship of the metabolite bioassay response to all of these measures of susceptibility in which greater sensitivity to the metabolites is related to greater resistance. However, this is not a qualitative relationship which is a characteristic of the discontinuous disease model (18,21), but a quantitative one in which the correlations of metabolite bioassay response to disease susceptibility are only modest, indicating the presence of other important factors affecting susceptibility.

Whether these tests reveal any information on the pathogenic capabilities of the fungal isolates is doubtful. There was no clear distinction between isolates in these bioassays. The five isolates were tested for canker-forming capabilities (14). One isolate, 208-6, had lost the ability to form cankers in this type of artificial inoculation system, yet it was one of the stronger metabolite producers as measured by these bioassays. Isolate 208-6 produced cankers vigorously in the original test (12); the loss of virulence observed in subsequent tests (10,14) was not related to loss of ability to produce detectable toxic metabolite activity in culture. Indeed, this isolate was the second most vigorous metabolite producer in grain culture (Fig. 5). For this isolate, other factors were clearly more closely related to canker formation than metabolite-producing ability as measured by these tests.

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