Tissue Culture and Leaf Spot Bioassays as Variables in Regression Models Explaining *Hypoxylon mammatum* Incidence on *Populus tremuloides* Clones in the Field

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ABSTRACT

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Regression models were used to interpret the relationships among sensitivity of aspen to metabolites produced by *Hypoxylon mammatum*, disease incidence in the field, and several other clone and site variables. Twenty-nine naturally occurring aspen clones in central New York were intensively surveyed. Dormant buds of 10 clones representing the range of variation in infection were cultured and bioassayed with culture filtrates of *H. mammatum* to compare with bioassays of leaves collected in the field. Bioassay systems based on tissue culture and field collections demonstrated the occurrence of clonal differences in response to metabolites produced by *H. mammatum*. High positive correlations among bioassays, with various fungal isolates, supported the conclusion that clonal response to metabolites is genetically controlled. However, low correlations of toxin assays with various measures of disease did not support the theory of a direct relationship between disease incidence and toxin assays. Rather, an interaction of the toxin assay with other clonal and site variables in regression models was required to explain the variation in disease incidence in the field.

The role in disease of metabolites produced by *Hypoxylon mammatum* (Wahl.) Mill., the causative agent of hypoxylon canker, remains undetermined (22). Hubbes (21) first noted that mycelial plugs of *H. mammatum* inhibited cambial growth. He attributed this to secretion of a toxin. Later, Schipper (25) reported that culture filtrates of the fungus were necessary to induce infection of aspen (*Populus tremuloides* Michx.). French (16) proposed that the metabolites could be used in a leaf spot bioassay to screen aspen for resistance to hypoxylon canker. His results suggested a direct relation between canker incidence and toxin sensitivity. Bruck and Manion (12), using the leaf spot bioassay, demonstrated a strong correlation between lesion diameter induced by the metabolites and disease incidence ($r = 0.92$). However, Ehrenshaft (14) could not reproduce Bruck and Manion's work, obtaining a negative correlation of $r = -0.52$. Pinon (23), doing similar work in Europe with *Populus tremula* L., found some correlation between reaction to toxin and natural disease incidence but admitted the occurrence of exceptions at the clonal level. More recently, Griffin and Manion (18) suggested that the leaf puncture bioassay does not measure pathotoxins per se but measures the ability of aspen clones to respond to elicitors. Their conclusion came from the finding that the culture filtrate bioassay yielded negative correlations with disease incidence and canker length and positive correlations with callus frequency.

This confusion has led to the exploration of new ways of describing the role of metabolites released by *H. mammatum*. Wann (29) first tried using a tissue-culture system to provide additional control of environmental factors in a bioassay; his results yielded qualitative observations that were not amenable to data analysis.

This study tests the hypothesis that sensitivity of aspen to metabolites produced by *H. mammatum* is related to hypoxylon canker incidence. To evaluate the environmental and genetic effects on responses of aspen to toxic metabolites, we tested tissue-cultured plantlets and field-collected leaves of naturally occurring aspen clone. The relationships among different measures of disease incidence in the field and an array of clone and site variables provided a foundation for comparison with toxic metabolite responses. Our objectives were to develop a quantifiable bioassay of aspen clone response to metabolites using a tissue-culture system and to assess the relationships among bioassays and various clone and site variables. A preliminary report of this study has been presented (11).

MATERIALS AND METHODS

Selection and assessment of aspen clones in the field. The characteristics outlined by Barnes (9) were used to select 29 naturally occurring aspen clones with at least 50 stems from four sites in central New York State. Although not strictly random, these clones, of 18 to 60 years of age, were assumed to be a representative sample of abandoned field succession populations from this region. The incidence of hypoxylon canker was determined during the winter by examining a total of 1,976 aspen stems for the characteristic symptoms and signs of *H. mammatum*. These included mottled dark and light dead area surrounded by yellow to orange bark and perithecial stroma and/or conidial pillars of *H. mammatum*. Cankered trees were recorded as living or dead and upper or lower cankered depending on the position of the canker with respect to the lowest living branch. The diameter at 1.4 m (DBH) of each stem over 2 cm provided the information for calculating the basal area (BA) in aspen and percent basal area of other species (OS). Each stem was assigned a crown class (8), and the percent of aspen in the codominant class (C) was determined. The percent crown cover (CC) for the plot was estimated and the density of aspen (D) was expressed as number of live stems per hectare. Average growth rate (GR) was determined from increment coring of four healthy dominant stems in each clone. Height measurements of these four stems provided a height in relation to age approximating site index (SI) (20). The percent slope of the site (S), the exposure (E) rated 1 to 5 with 1 being most exposed (15), and aspect (A) transformed as described by Beers et al (10) to express 0 as southwest and 2 as northeast were determined for each clone.

Selection and propagation of aspen clones in vitro. The 29 naturally occurring clones were separated into nine distinct classes of disease incidence (percent of living and dead trees with cankers in a clone) by a Scott-Knott test (17). Two clones were randomly

selected from each class for a total of 18 clones. Dormant buds from each clone were collected and washed in distilled water for 30 min. The buds then were transferred to 25 ml of a 5% sodium hypochlorite solution containing 2–3 drops of Triton X-100 for 15 min. The bud scales were subsequently removed, and the bud meristem with a few juvenile leaves were sterilized in a 1% sodium hypochlorite solution for 5–10 min. After the bud explants were washed four times in sterile distilled water, they were cultured at 22 C on aspen culture medium (1,2) supplemented with gentamycin (30 mg/l) and tetracycline (40 mg/l). The photoperiod was 16 hr under cool-white fluorescent lights with an intensity of 2,000 lx measured with a Spectronic 20 Photometer (Bausch & Lomb Inc., Rochester, NY). Of the original 18 clones selected, only 10 proliferated. The first shoots appeared after 5 wk. Subculturing the plant material every month promoted shoot development. After 5 mo, a sufficient number of shoots was obtained.

Bioassays. For bioassay of leaves collected from the 25 clones in the field, toxic culture filtrates and procedures of a previous study (18) were used. Five culture filtrates were tested on a total of 50 leaves based on May and August collections from five stems per clone.

For assays of 10 tissue-cultured clones, three additional filtrates were prepared from recently isolated cultures of *H. mammatum* grown on basal medium supplemented with 5 g/L of glutimate (19). The culture filtrates were prepared as before (18) to 1% of the original volume in 70% methanol, adjusted to pH 7.0 with KOH, and sterilized using 0.22-μm filters. The bioassay involved two procedures. The first was very similar to the leaf spot bioassay. A leaf attached to the plantlet was wounded with a minutin insect pin and a 2-μl drop was placed over the wound. In the second procedure, a minutin insect pinprick was made directly into the stem of the plantlet, and a 2-μl drop was placed over the wound. The plantlets were carefully removed from the culture medium for the application of toxin to leaf or stem and then returned to fresh medium. The two procedures were followed for all three culture filtrates. Sterile culture filtrate medium treated as above was used for controls. This experiment was repeated four times for all 10 clones. Plantlet response was recorded after 72 hr.

Regression models. Disease incidence was thought to be potentially predictable by either clonal response to metabolites alone or clonal response to metabolites and a number of clone and site variables. These relationships were tested for the 29 clones with four measures of disease resistance in the field and all of the clone and site variables listed above. For the 10 tissue-cultured clones, we tested six field variables: growth rate, site index, basal area of aspen, slope of the site, transformed aspect of the site, and exposure of the site. The regression models were selected by the stepwise regression procedure provided by the computer program package of the Statistical Analysis Systems Institute, release 82.3 (24).

RESULTS

Bioassays. The range of necrotic responses caused by culture filtrates on tissue-cultured plantlets is shown in Figure 1 for leaf and stem applications. In all cases, the necrosis expanded from the application point. No control plantlets treated with sterile culture filtrates exhibited necrotic responses and therefore were not included in the calculations. Table 1 shows a gradient of clonal responses to toxic metabolite exposure based on the rating system explained in Figure 1. Correlations among the responses in Table 1 plus the field-collected leaf spot bioassay were quite high (Table 2).

Regression models. Simple correlation analysis demonstrated very little relationship of toxin assays and percent hypoxylon canker in both field-collected leaves and tissue-culture tests ($r = 0.16$ and 0.02, respectively). Percent hypoxylon-caused mortality and percent hypoxylon incidence in the upper crown were likewise not correlated with the toxin assay of field-collected leaves ($r = 0.01$ and 0.06, respectively). Toxin assay of field-collected leaves was significantly correlated with cankering in the lower portion of the stem ($r = 0.57$, $P = 0.0013$).

The four measures of hypoxylon disease in the field were variously related. Total hypoxylon incidence in relation to mortality, lower cankers, and upper cankers had correlations of $r = 0.90$, 0.62, and 0.51, respectively. Percent incidences of upper and lower cankers were not correlated ($r = 0.02$).

Attempts were made to explain disease incidence by combining toxin responses with clone and site variables in regression models shown in Table 3. Analysis of all clones and site variables with the stepwise regression procedures entered percent of other species, density of aspen, percent codominant aspen, growth rate, and lesion diameter of field-collected leaves. With the tissue-culture assay of 10 clones and a selected set of clone and site variables, the stepwise regression procedure entered the variables growth rate, toxin assay, transformed aspect, and approximate site index, producing increasing R^2 values of 0.29, 0.48, 0.70, and 0.91 as each variable was added to the model. The other three variables added little to increase R^2 and therefore were discarded to reduce bias. The standardized contribution of the four variables used in the tissue-culture model is presented in Figure 2. Three additional regression models were derived to predict hypoxylon mortality, lower stem canker percent, and upper crown canker percent (Table 3).

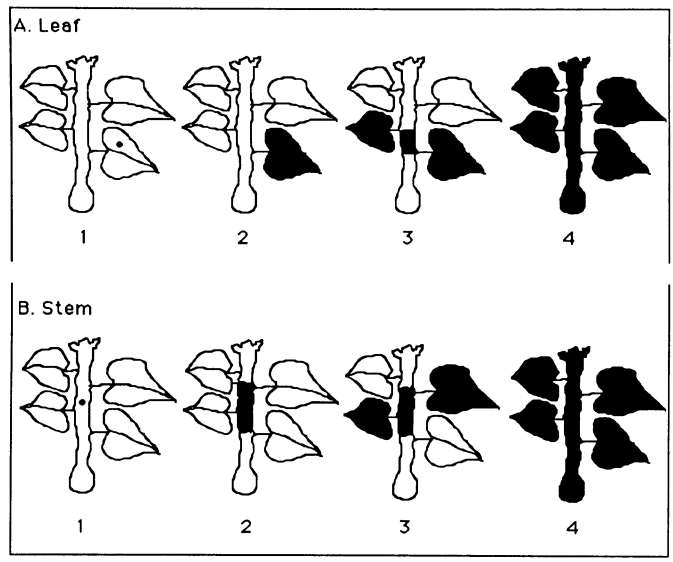

Fig. 1. Range of symptoms observed on *Populus tremuloides* plantlets following **A,** leaf injection and **B,** stem injection of *Hypoxylon mammatum* metabolites. The number below each plantlet indicates the rating given based on the symptoms observed. (A zero was attributed if no visual symptoms were present.)

TABLE 1. Average responses of plantlets of 10 clones of *Populus tremuloides* to metabolites from three isolates of *Hypoxylon mammatum* using stem and leaf application procedures

Clone	Overall[a]	Stem[b]	Leaf[b]	Isolates[c]		
				50-75-1	50-75-5	50-99-4
D10	3.8	3.9	3.8	3.9	3.8	3.9
B40	3.5	4.0	3.0	4.0	3.6	2.9
B30	2.1	2.0	2.3	3.0	1.9	1.5
C50	2.0	1.2	2.9	3.4	1.9	1.4
A10	1.9	2.1	1.7	3.1	1.5	1.0
B80	1.5	1.2	1.8	3.0	1.1	0.3
C30	1.1	0.7	1.6	2.1	0.4	1.0
B10	0.5	1.1	0.1	1.5	0.1	0.0
A40	0.5	0.8	0.3	0.4	0.0	1.1
B70	0.3	0.0	0.6	0.0	0.7	0.0

[a] Average of in vitro leaf and stem applications and all three isolates based on a 0–4 rating scale (n = 24).
[b] For all three isolates based on a 0–4 rating scale (n = 12).
[c] For in vitro leaf and stem applications based on a 0–4 rating scale (n = 8).

DISCUSSION

Responses presented in Table 1 express a wide range of clonal sensitivity to the metabolites. The same data show that fungal isolates used in this study produce metabolites with different toxicities. However, high positive correlations relating these differences were consistently observed (Table 2). These correlations remain high when the values are compared with the leaf spot bioassay where different fungal isolates were used. This suggests that clonal sensitivity to the metabolites is under genetic control and that both the tissue culture and the leaf spot bioassay can be used as measures of this trait. However, if the sensitivity is under genetic control and is related to the susceptibility of the clone to hypoxylon canker, it seems contradictory to observe low correlations between the bioassay and disease incidence. This may indicate that the theory of direct relationship between toxin assay and disease incidence, as put forth by some authors (26,27), is too narrow in its conceptualization because it may overlook some important factors involved in the overall mechanism. Wallace (28) has expressed the same concerns, pointing out that a disease that can be explained by a single factor is a rare occurrence in nature. This would explain the discrepancies obtained in previous experimental results using a toxin assay as the sole explanatory factor.

The regression models corroborate the theory of a more complex system. The involvement of clone and site variables in hypoxylon canker development has already been proposed by several authors (4,6,13). Some studies have tried to associate site index (4,13), growth rate (4), other species (4,5,7), and stand density (3,4,13) to hypoxylon canker incidence. The input of these variables in the models presented here supports their role as interacting factors in the disease development.

TABLE 2. Correlation coefficients[a] among inoculation procedures and isolates based on the average responses of plantlets and detached leaves of 10 clones of *Populus tremuloides* exposed to metabolites of *Hypoxylon mammatum* from three isolates

	Overall	Stem	Leaf	Isolates 50-75-1	Isolates 50-75-5	Isolates 50-99-4	LSB[b]
Overall	1.00	0.95	0.94	0.89	0.97	0.91	0.88
Stem		1.00	0.78	0.79	0.92	0.89	0.76
Leaf			1.00	0.88	0.91	0.83	0.91
50-75-1				1.00	0.81	0.66	0.75
50-75-5					1.00	0.88	0.87
50-99-4						1.00	0.86
LSB							1.00

[a] Values above 0.77 significant at $P \leq 0.01$ and values above 0.65 significant at $P \leq 0.05$.
[b] Leaf spot bioassay.

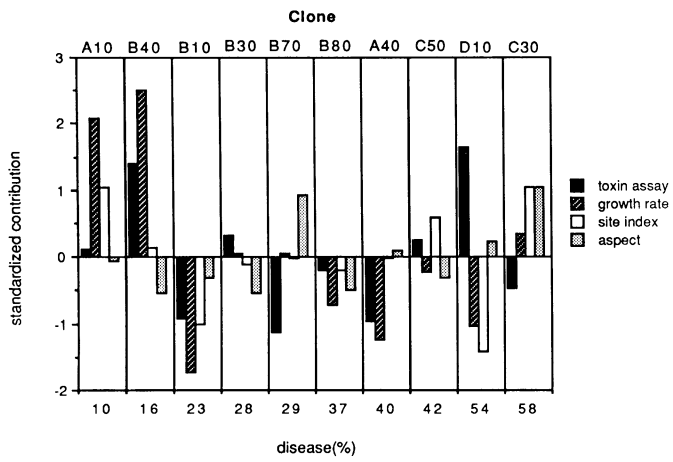

Fig. 2. Tissue-culture toxin assay and field growth rate, site index (approximate), and aspect (transformed) variables normalized and weighted by regression model parameters for 10 *Populus tremuloides* clones ranked from low to high percent hypoxylon canker incidence.

TABLE 3. Standardized multiple regression models for the relationships among various measures of hypoxylon incidence in aspen clones in relation to tissue-culture and leaf spot toxic metabolite bioassay responses and various clone and site variables

Models[a]	R^{2b}
Total percent disease = -1.37 GR + 0.95 TT + 0.58 A + 0.79 SI	0.91**
Total percent disease = -0.96 OS $-$ 0.60 D $-$ 0.53 C $-$ 0.47 GR + 0.39 LT	0.62*
Percent mortality = -0.96 OS $-$ 0.60 C $-$ 0.49 CC + 0.44 E	0.67**
Percent lower cankers = $-0.67\ D^2 + 0.50\ LT^2 + 0.47\ OS^{-0.5} - 0.46 \ln (SD)^2$	0.69**
Percent upper cankers = $0.90\ SI^{-2} - 0.48\ S^{-0.5} + 0.32\ BA^{0.5} - 0.29\ C^2$	0.75**

[a] TT = tissue-culture toxic metabolite bioassay; LT = leaf spot toxic metabolite bioassay; GR = growth rate (mm yr^{-1}); A = transformed aspect; SI = approximate site index; OS = other species (%); D = density of aspen (stems ha^{-1}); C = aspens in codominant crown class (%); CC = crown cover (%); S = slope (%); BA = basal area of aspen (m^2); SD = standard deviation of aspen diameter at 1.4 m; E = exposure.
[b] Significance of models: ** is $P<0.001$, * is $P<0.01$.

The various measures of disease in the field resulted in different models because the measures of disease were not necessarily correlated. Total disease incidence is a composite of mortality and upper and lower cankered trees and therefore best reflects the disease potential of the clone and site. The models based on the other measures of disease demonstrate how interpretation may change if one is not careful to assess the total disease incidence within the clone. It is inappropriate to assign major significance to the selected variables particularly because the variables changed from one model to another. These models are most instructive in pointing out the complexity of the system and specific interpretation is a matter of individual judgment.

For example, the role of metabolites as toxins or elicitors remains unsettled. From Figure 2, it appears that our data support the hypothesis of toxic properties. Assuming that high growth rate improves natural resistance, disease incidence generally can be rationalized as a function of toxic properties. At the lowest disease incidences, high growth rates offset average to high susceptibility to the toxin to confer a high resistance to the clones. On the other hand, if the growth rate is low and the toxin susceptibility is high, one should expect a high disease incidence as demonstrated by the 54.4% incidence level for clone D10. However, all examples do not necessarily support toxic properties as clearly. Pinon (23), working with various poplar species, concluded that the metabolites generally function as toxins, but comparisons among clones within species were not necessarily consistent with the toxin interpretation. Nevertheless, whether the metabolites act as toxins or elicitors, it can be concluded that the extent to which they relate to disease incidence is highly dependent on the growing conditions to which the clones are exposed. This indicates that a toxin bioassay cannot be used as a single variable in screening for hypoxylon canker resistance; future efforts therefore should aim at understanding the exact role of environmental variables in the development of the disease.

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