Canker Elongation, Branch Death, and Callus Formation as Resistance or Susceptibility Responses in *Populus tremuloides* and Virulence or Avirulence Characteristics of *Hypoxylon mammatum*

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ABSTRACT

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Five single-ascospore isolates of *Hypoxylon mammatum* were tested for virulence against nine clones of *Populus tremuloides* planted in a randomized complete block experiment. Measurements of canker length, branch death frequency, and callus formation frequency were made over a 16-mo period encompassing two growing seasons. There were significant differences among clones and isolates for all three measurements. The fungal isolates accounted for much more of the variation in canker lengths than did the aspen clones, while the fungal isolates and aspen clones varied to about the same extent in branch death and callus formation. Branch death and callus formation were mutually exclusive on a given canker, but showed no significant correlations in frequencies among the clones and isolates. Canker length showed a moderately negative correlation with callus frequency and a moderately positive correlation with branch death. These results are consistent with the hypothesis that branch death and canker length are indicators of virulence in the fungus. The varying degrees of interrelationships among canker length, branch death, and callus formation indicate the complexity of the host-pathogen interaction. There was considerable independence of these characteristics among both the aspen clones and the fungal isolates.

Hypoxylon mammatum (Wahl.) Miller causes a stem canker in aspen (*Populus tremuloides* Michx.), and probably is its most significant disease (2,8). Genetic disposition of the host towards the pathogen may be one of the major factors affecting disease incidence (3), suggesting that clone selection and breeding for greater resistance would be useful in improving the crop potential of aspen. Reasonable progress in a selective breeding program for a relatively long lived plant requires identification of resistance traits in young plants. Resistance to *Hypoxylon* can be expressed in several ways during the course of canker development. Disease incidence in nature is the result of the combined effects of the success of the pathogen in the infection court and the ability of the pathogen to maintain its presence in the host after establishment. Damage from the disease depends not only on these two factors, but also on the extent to which the pathogen can extend the cankers into the main stem causing cambium death, wood decay and eventual death or breakage of the tree. Previous investigations have used canker length as a measure of virulence of the pathogen or resistance in the host (1,5–7). Callus formation and branch death have also been studied as possible mechanisms of disease resistance (7,10). All of these studies have been relatively short term (3–10 wk) and have not controlled site factors that may affect the response (5–7).

In the present study a uniform replicated experimental planting of nine aspen clones inoculated with five isolates of *H. mammatum* is used to determine what relationships, if any, exist among canker length, callus formation, and branch death; and to attempt to clarify the relative importance of host variation and pathogen variation in these responses, to better understand their relation to host resistance (or susceptibility), and to serve as measures of pathogen virulence or avirulence.

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MATERIALS AND METHODS

Nine aspen clones growing in a garden plot at the Genetics Field Station, Tully, NY, were inoculated with five single-spore isolates of *H. mammatum*. Five of the clones had been tested in the wild (7), and were propagated from root sprout cuttings for this test. Four additional clones also from Onondaga County, NY, were similarly propagated. Two-year-old rooted cuttings were outplanted in 1973 and 1974 in a randomized complete block design. Each of the three replications of the blocks contained nine ramets of each clone planted in a 3 × 3 square with 3 m between trees. Border tree plantings around the total planting were used to minimize edge effects.

Five single ascospore isolates of *H. mammatum*, originally isolated by French (5) from the clones, were maintained on 2% malt agar at 4 C with annual subculturing. Mycelium growing on sterile grain was placed in 6-mm-diameter circular wounds in the bark of 2-yr-old branches. The procedures are similar to those previously described (10) except that Parafilm was used to cover the inoculation. Three branches on each of six trees in a block were inoculated. Inocula of individual isolates (or sterile grain controls) were inserted one per branch on three trees of each clone in each of the three blocks for a total of nine inoculations per clone per isolate. Inoculations were made in early July 1981 and cankers were measured and scored for callus formation and branch death after 4, 12, and 16 mo.

The analysis of variance was by the general linear models procedure of Statistical Analysis Service (SAS Institute, Inc., Cary, NC; Release 79.6). The analysis of variance was done according to a factorial model with interactions as indicated in the Results section. The squared semipartial correlations were calculated as the ratio of the sums of squares for each source of variation with the total sums of squares for the factorial model (4). The source of the error is the within-class variation. Frequency data were analyzed by the G-test of homogeneity and the simultaneous test procedure for G as described in Sokal and Rohlf (9).

RESULTS

Canker lengths. Production of cankers on the inoculated trees varied considerably among the isolates. No cankers were produced

on the control inoculations. Only four of the inoculations with isolate 208-6 remained uncallused at 4 mo and two remained uncallused at 12 and 16 mo. While callus formation is clearly a resistance mechanism, other mechanisms of resisting or retarding the progress of canker development may exist that are independent of visible callus formation. For this reason we examined canker length by considering only inoculations that produced expanding cankers.

TABLE 1. F test significance probabilities and squared semipartial correlations (r^2) of an analysis of variance of canker lengths measured at 4, 12, and 16 mo for five isolates of *Hypoxylon mammatum* inoculated into nine clones of *Populus tremuloides*. Only inoculations producing uncallused cankers were included

	4 mo		12 mo		16 mo	
Source	Prob.	r^2	Prob.	r^2	Prob.	r^2
Clone	0.0001	0.16	0.0001	0.10	0.14	0.03
Isolate	0.0001	0.30	0.0001	0.28	0.0001	0.22
Clone × isolate	0.0001	0.10	0.0002	0.10	0.34	0.07
Plot	0.002	0.02	0.0003	0.04	0.42	<0.01
Replicates	0.59	<0.01	0.52	<0.01	0.26	<0.01
Total model	0.0001	0.57[a]	0.0001	0.53	0.0001	0.33[a]

[a] For the total model this is the coefficient of determination which is usually symbolized by R^2

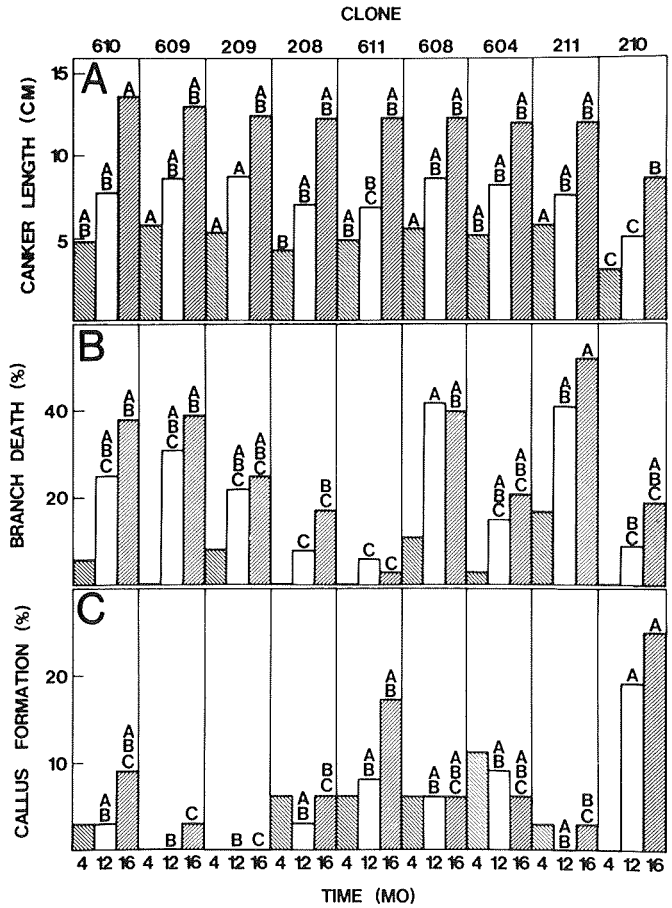

Fig. 1. Variation among nine clones of *Populus tremuloides* for the mean canker development responses to the isolates of *Hypoxylon mammatum* at 4, 12, and 16 mo. Bars for the same time period with the same superposed letters are not significantly different, $P = 0.05$, according to Tukey's studentized range test for canker length and the simultaneous test procedure with G for callus and branch death. **A,** Means of canker lengths for all the cankers producing expanding cankers. Callused inoculations were not included. **B,** Branch death frequencies for the inoculations with the four virulent isolates of *H. mammatum*; isolate 208-6 and the controls were not included. **C,** Callus formation frequencies for the inoculations with the four virulent isolates of *H. mammatum*. Inoculations with isolate 208-6 and the controls were not included.

Analysis of variance of canker lengths revealed significant differences among clones, isolates, and clone × isolate interactions at 4 and 12 mo, but only for isolates at 16 mo (Table 1). There were also significant plot differences at 4 and 12 mo, but very little of the variation was accounted for by the plots. By 16 mo, the only significant differences were for isolates. The squared semipartial correlations (r^2) indicate that the isolates were the greatest contributors to variation in canker length. The clones accounted

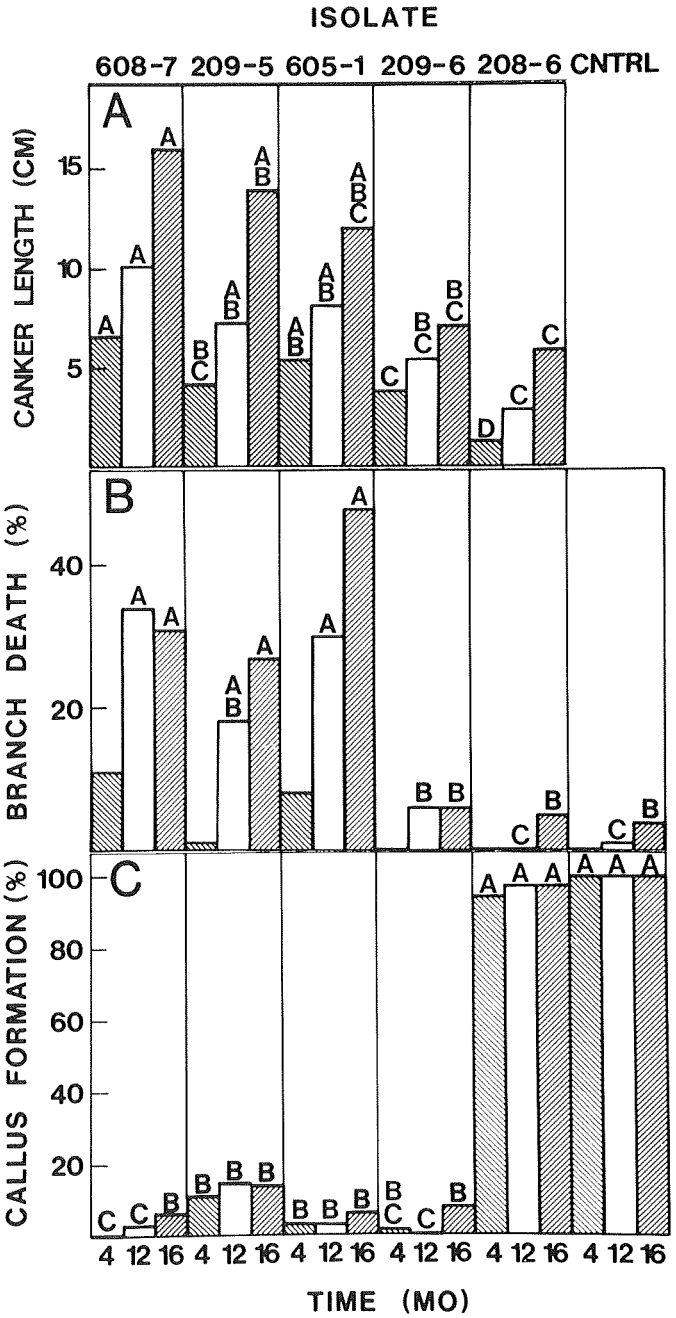

Fig. 2. Variation among the five isolates of *Hypoxylon mammatum* and controls (CNTRL) for the mean canker development responses to the nine clones of *Populus tremuloides* at 4, 12, and 16 mo. Bars for the same time period with the same superposed letters are not significantly different, $P = 0.05$, according to Tukey's studentized range test for canker length and the simultaneous test procedure with G for callus and branch death. **A,** Means of canker lengths for all inoculations that produced expanding cankers. Controls not shown because no cankers developed. Isolate 208-6 had produced only four cankers at 4 mo and only two of these remained at 12 and 16 mo. **B,** Branch death frequencies of the inoculations of the five isolates and controls (CNTRL) onto the nine clones of *P. tremuloides*. **C,** Callus formation frequencies of the inoculations of the five isolates and controls (CNTRL) onto the nine clones of *P. tremuloides*.

for much less of the variation. A substantial decrease of r^2 values from 4 to 16 mo for the clones, the isolates, and the total model indicate that other factors not accounted for had an increasing effect on variation in canker length in this experiment as time progressed.

The differences among clones is described by Tukey's studentized range test (Fig. 1A). The differences were not consistent except that clone 210 always had the shortest cankers. By 16 mo, seven of the clones were indistinguishable. Only the extremes, clones 610 and 210, were significantly different by this test. This difference was detected by Tukey's test even though the analysis of variance indicated no significant difference among clones. Greater differences are seen when canker lengths are compared by using Tukey's studentized range test across the isolates (Fig. 2A). When averaged across all nine clones, isolate 608-7 consistently had the longest cankers, and isolates 209-6 and 208-6 consistently had the shortest.

A comparison of the clones with each isolate is presented in Fig. 3. Isolate 208-6 is not included in this analysis because it produced too few cankers. The clones showed quite different patterns of canker elongation depending on the isolate. Isolate 608-7 produced the longest cankers of all the isolates on all but two clones. Isolate 209-6 produced cankers that were quite uniform among the clones and were the shortest cankers of all the isolates on each clone. Isolates 209-5 and 605-1, with intermediate canker lengths, showed significant variability among the clones and quite different rank orders of clones by canker length. Clone 210 showed the shortest canker lengths with three of the four isolates. No clone consistently had the longest canker lengths.

Comparison of the canker growth rates exhibited by the isolates for the three time periods revealed some interesting variation (Table 2). The middle growth period was the slowest, being largely during the winter-spring period (November through June), while the two summer periods showed the most rapid growth. Two of the isolates, 209-6 and 605-1, showed similar growth rates during the two summer periods, but isolates 209-5 and 608-7 grew considerably faster the second summer with 209-5 being >2.5 times faster the second summer compared to the first.

Branch death. Branches were recorded as dead when the leaves distal to the inoculation point had become brown or had failed to emerge or when they were broken off at the canker. In some cases, the proximal portion of the branches had also died, but usually only the distal portions were obviously dead. Only the four virulent isolates were considered when comparing branch death frequencies among the clones (Fig. 1B). For statistical analysis they were assigned a value of 0 if alive and 1 if dead.

The *G*-test for heterogeneity revealed significant differences among clones and among isolates for the 12- and 16-mo measurement times (Fig. 1B). This test is not useful with the 4-mo data since too many cells in the frequency table are 0. The rank order of clones is essentially alike for all three measurement times except for clone 609, which had no branch death at 4 mo but was third highest at 12 and 16 mo. Clones 211 and 608 consistently had the highest frequencies and clone 611 consistently had the lowest (Fig. 1B).

For comparison of the isolates, the controls and 208-6 were included because they reveal the low level of branch death from uncontrolled causes and natural pruning (Fig. 2B). Isolate 209-6 was similar to 208-6 and the controls except that it had a slightly, but significantly, higher level of branch death at 12 mo. The other three isolates all caused considerably higher incidence of branch death (Fig. 2B).

Callus frequency. Callus formation on the inoculations varied from no visible callus to slight callus ridges (which prevented girdling) along the bottom edges of the cankers to completely closed wounds. Only the latter were scored as callused. For statistical analysis, callused inoculations were scored as 1, uncallused as 0.

The *G*-test for heterogeneity revealed significant differences among clones and among the virulent isolates, but not at all measurement times (Fig. 1C). The effect of time on the development of callus was most dramatic for clone 210, which had callus only with the control and isolate 208-6 inoculations at 4 mo, but had the highest frequencies of callus at 12 and 16 mo. It should also be noted that the apparent decrease in callus frequency for clone 604 was due to the loss of branches during the course of the experiment and not to expansion of cankers from previously callused inoculations. In no case did any callused inoculations develop cankers at a later time. Clone 209 was unique in never producing any callus against isolates capable of producing cankers. The most important differences in callus frequencies were between clones 210 and 611 with the highest levels and clones 209, 211, and 609 with the lowest levels of callus.

The principal difference among the isolates was for 208-6 and the canker-forming isolates (Fig. 2C). By 12 mo, only two of the 80

TABLE 2. Elongation rates of cankers caused on *Populus tremuloides* by *Hypoxylon mammatum* for the three-time intervals between measurements

Isolate	Growth rates[a] ($mm \cdot mo^{-1}$)		
	Summer 1981	Winter-spring	Summer 1982
209-6	6.4	2.1	5.5
209-5	7.5	3.8	19.5
605-1	10.8	3.4	11.4
608-7	13.4	4.4	17.3

[a] Mean canker length of isolates corrected for control lesion length for nine clones based on 80 inoculations for each isolate.

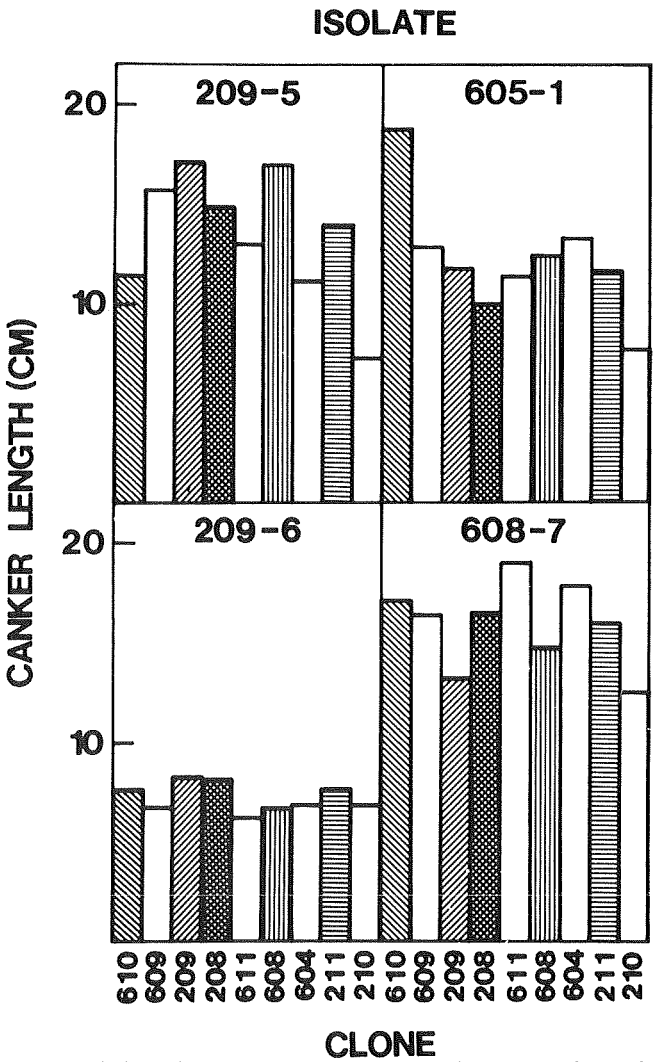

Fig. 3. Variation of canker lengths among the nine clones of *Populus tremuloides* for each of the four virulent isolates of *Hypoxylon mammatum* at 16 mo. Values indicated are the means of those inoculations that produced expanding cankers for each clone-isolate combination.

inoculations with isolate 208-6 were not callused. Isolate 209-5 had consistently the most callused inoculations of the virulent isolates, but by 16 mo, it was not significantly different from the other three. Isolate 209-6, which consistently produced the shortest cankers, had a low callus frequency, but had cankers with large surrounding callus ridges at 16 mo. These cankers were not closed and, therefore, were recorded as uncallused.

DISCUSSION

The analysis of variance of the canker lengths indicates that these garden experiments largely eliminated site factors as sources of variation in studying host-pathogen interaction in this system. Although there were significant plot differences between the three replicate plots in some cases, the very low values of r^2 for the plots indicates that plot differences are not important in measuring the host-pathogen interactions in this experiment. Previous inoculation of clones in the wild could not separate clone from site variation (6,7).

Some significant differences in canker length between clones were apparent at 4 and 12 mo, but the fact that most of these differences disappeared by 16 mo suggests that this is not a reliable measure of resistance to *Hypoxylon* among the clones. The comparison of averages of the clones across the collection of isolates used in this study conceals variation in the measurements exhibited by specific clone-isolate combinations. This type of variation is illustrated with the canker length data at 16 mo (Fig. 3). However, the general response of the clones represented by the average of many isolates is probably more meaningful since aspen grows as clones in nature and is subject to a variety of natural inoculum from a highly variable pathogen (1,7, and these results). However, the fact that short cankers were consistently formed on clone 210 suggests further research may show canker length to be a valid characteristic of host resistance.

The greater variation in canker length among the isolates and the consistency of the ranking of the isolates indicates that canker elongation is an important source of variation in the fungus. The variation in canker growth rates of the fungal isolates also indicates the complexity of the patterns of canker development that are involved in this response. Clearly, any single response time for observation of canker length does not give a true picture of the interactions involved. The seasonal growth response of the cankers observed in these experiments is comparable with the observation of French (5) that inoculations early in the summer produced longer cankers than later inoculations. But the fact that the isolates vary so much in their growth rates from year to year indicates that single or short-term measurements of canker length are misleading.

Branch death frequencies in a previous study (10) and in the present study, show a greater degree of variability among the clones than did canker lengths. The high frequencies of branch death, 40 to 60%, on some clones and near absence on other clones and similar ranges of variation for the fungal isolates indicate that genetic variation is about the same in the host and the pathogen. Whether early branch death is a resistance mechanism or a sign of susceptibility is not clear at this time. It could act to prevent the pathogen from moving into the main stem by promoting self pruning. It could also cause the elimination of the pathogen by promoting the entry of saprobes which compete more effectively in dead tissue. For example, *Cytospora* fruits very quickly on the dead branches and the typical canker caused by *Hypoxylon* is rapidly obliterated. On the other hand, the fairly strong positive correlation of branch death with canker length ($R = 0.43$ to 0.83) indicates that branch death is related to the virulence of the fungus.

On the basis of half-sib families, it was suggested that branch death is a heritable response which was variably affected by inoculum source (10). The fungal isolates in our garden test likewise show variable branch death effects. Isolates 209-6 and 208-6 do not differ greatly from controls. The other three isolates produced 30–50% branch death in 16 mo. As with canker length, the clone and isolate means obscure individual variation which ranged from 0 to 100% for isolate 605-1 but only from 0 to 22% for isolate 209-6 on various clones.

Callus formation was interpreted as a resistance mechanism controlled by a single gene as well as by more complex genetic systems (10). Callus response frequency was isolate-dependent, but some family isolate combinations responded quickly while others developed callus after a period of canker elongation (10).

In the present tests, callus frequencies showed considerable variability among the aspen clones, though not as great as with branch death. Callus formation is more easily justified as resistance in the host than branch death as a mechanism for walling off the pathogen. This was especially evident with isolate 208-6 which seems to have lost its virulence. This isolate produced active cankers in nearly all of its inoculations in five of these same aspen clones in the wild (7) but produced very few cankers when inoculated on aspen in Michigan a few years later (5). The cankers produced by isolate 209-6 had developed heavy callus ridges by 16 mo, but they had not closed the cankers. However, the shortness of the cankers of this isolate indicates that active processes limiting the invasion of this isolate were functioning. Callus formation was part of this response. Isolate 209-5 generated callus response in 18% of the inoculations, but comparison of callus responses of the individual clones to this isolate showed a range from 0 to 62%. As previously observed (10), the callus response in family-isolate combinations suggests a strong genetic control mechanism.

Branch death and callus formation showed a significant mutually exclusive interaction at the individual inoculation. After 16 mo, 307 of the inoculations of the four virulent isolates remained and could be scored. Of these, 83 showed callus formation alone, 22 showed branch death alone, 199 showed cankers with neither branch death nor callus, and only three had both callus formation and branch death. These three may represent branches dead from causes not related to the disease process. The correlations between callus and branch death are weakly negative ($R = -0.34$ to -0.51) or nonsignificant. Thus, while there is a strong interaction at the individual inoculation, there is very little correlation between the propensities of the clone-isolate pairs to yield either branch death or callus formation. The mutually exclusive interaction is a physiological interaction at the branch and not a genetic characteristic of the particular clones and isolates.

The moderately strong positive correlations of canker length with branch death ($R = 0.43$ to 0.83) are consistent with the hypothesis that branch death and canker lengths are indicators of virulence in the isolates. The negative relationship between callus formation and canker length supports this, but the general lack of significant correlations between callus formation and branch death as well as the moderate levels of correlations between the other responses suggests that these are independent measures of virulence and are not highly redundant.

These varying degrees of interrelation between canker length, branch death, and callus formation are indicative of the complexities of the host-pathogen interaction. While the various facets of canker development interact with each other, there is considerable genetic independence of these characteristics among both the aspen and the fungal populations.

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