# Hypovirulence, Vegetative Incompatibility, and the Growth of Cankers of Chestnut Blight

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## ABSTRACT

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American chestnut trees were inoculated with pairs of strains of *Endothia parasitica*, and the rate (*b*) of the expansion of the area of cankers was measured. Hypovirulence (H) agents in one strain of the pair decreased *b* markedly if both strains were in the same vegetative compatibility (v-c) group. With increasing numbers of v-c alleles different between the pair, the decrease in *b* became less and less until the decrease became insignificant when different alleles at five v-c genes separated the pair of strains. Although various strains in different v-c groups were used, the results were consistent. French and Italian H agents had similar effects on *b*.

*Additional key words:* biological control.

Although *Endothia parasitica* (Murr.) And. has killed nearly every mature tree of *Castanea dentata* (Marsh.) Borkh. in the northeastern United States since the fungus appeared here in 1904, a similar epidemic beginning among trees of *C. sativa* in Italy in the 1930s is no longer a serious problem (10,11). The reversal in Italy was probably caused by the appearance of hypovirulent (H) strains of the pathogen containing viruslike agents that decrease pathogenicity (6).

Efforts to introduce the viruslike agents into American *E. parasitica* led to the discovery of vegetative incompatibility that inhibits transfer of the agents between some strains (1). Transfer occurs easily in vitro between strains in the same vegetative compatibility (v-c) group, but with varying degrees of difficulty (or not at all) between strains in different v-c groups (3). Seventy-seven v-c groups have been found among American strains, 68 among the 225 strains that were mass isolates from the stromata of cankers

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and nine among the progeny of crosses (Anagnostakis, *unpublished*). We found nine v-c types among 49 Italian mass isolates, and J. Grente has collected 22 v-c groups from several European countries (*personal communication*). Unfortunately "vegetative incompatibility (in fungi) will markedly reduce the spread of suppressive, cytoplasmic genetic elements, including viruses, from strain to strain in nature" (4). Since vegetative compatibility between strains requires that they have identical alleles at each of at least five v-c loci, it is possible to select strains that differ at 0, 1, 2, and 5 genes for v-c (2, and Anagnostakis, *unpublished*).

In the experiments reported here, we examined the effect of degrees of vegetative incompatibility on the growth of normal strains in chestnut following a second inoculation with either another normal strain or one that contained H agents from different sources.

## MATERIALS AND METHODS

The experiment tested the different rates of expansion of cankers caused by *E. parasitica* in field-grown American chestnut trees. The fungus was introduced in twin inoculations of six kinds, called treatments: S = two normal strains of same v-c type; FS = like S, but one strain containing French H agents; IS = like S, but one strain containing Italian H agents; D = two normal strains of different v-c type; FD = like D, but one strain containing French H agents; and ID = like D, but one strain containing Italian H agents. Comparisons among these six treatments showed the effect of H, its modification by a difference in v-c groups, the difference between the French and Italian H, and the effect of different v-c groups in the absence of H. To eliminate differences among trees from the test of treatments, the six treatments were all applied in random order up the side of a single tree (this is referred to as a set). The six treatments extended from 85 to 190 cm from the ground.

To increase the generality of the results, the treatments were replicated with a variety of inocula from several v-c groups (Table 1). Thus, set I was inoculated with strains 501 and 67 for treatment S, with 501 and 67 carrying French H for treatment FS, and with 501 and 67 carrying Italian H for treatment IS. Both 501 and 67 are in v-c group 10. Treatment D was inoculated with strain 155 in v-c group 40 and strain 67 in v-c group 10. In treatments FD and ID, strain 67 carried H. The v-c groups 10 and 40 are determined by different alleles at a single gene locus (Anagnostakis, *unpublished*).

The v-c groups of set II also differed at a single gene, but different strains were used to broaden the generality of the results. The v-c groups of sets III and IV differed at two v-c genes, but again different strains were used in III and IV. Finally, the v-c groups of sets V and VI differed at five or more v-c genes.

The six twin inoculations of sets I, II, IV, V, and VI were replicated three times (on three different trees). One replicate was missing in set III, and the sizes of the six missing cankers were estimated in the usual fashion for missing data, and the degrees of freedom for the error mean square was decreased by six. The total number of twin inoculations and subsequent cankers was $(6\times5\times3) + (6\times1\times2) + 6$ estimated cankers = 108.

Four of the strains of *E. parasitica* are deposited with the American Type Culture Collection: strain 42 is ATCC #38751, strain 6 is #22508, strain 67 is #38753, and strain 155 is #38755. The other strains were 110 from Hamden, CT; 500 and 501 from Hampton, CT; and 305 from Mt. Tom State Park, CT.

The H agents originated in strains 113 (French, ATCC #38771) and 420 (Italian, Jaune Régénéré type), and were transferred into strains 42, 6, 67, and 155 as previously described (3).

The American chestnut trees were sprouts from old roots on the Yale Golf Course in New Haven, CT. Their heights were approximately 4 m and their diameters at a height of 1.4 m averaged 8 cm.

On 19 May 1980, each tree side was inoculated in six places at intervals of about 21 cm. Inoculum was a 10-mm plug cut from a

TABLE 1. The vegetative compatibility (v-c) groups and strains of *Endothia parasitica* inoculated as six sets[a] into the trunks of American chestnut trees on 19 May and 2 June 1980

| Set | Treatments[b] | v-c Genes different between groups | v-c Groups and (strains)[c] 19 May | 2 June |
|---|---|---|---|---|
| I | S, FS, IS | 0 | 10 (501), | 10 (67†) |
| | D, FD, ID | 1 | 40 (155), | 10 (67†) |
| II | S, FS, IS | 0 | 40 (305), | 40 (155†) |
| | D, FD, ID | 1 | 10 (501), | 40 (155†) |
| III | S, FS, IS | 0 | 5 (110), | 5 (42†) |
| | D, FD, ID | 2 | 8 (6), | 5 (42†) |
| IV | S, FS, IS | 0 | 8 (500), | 8 (6†) |
| | D, FD, ID | 2 | 5 (110), | 8 (6†) |
| V | S, FS, IS | 0 | 10 (501), | 10 (67†) |
| | D, FD, ID | 5 | 5 (42), | 10 (67†) |
| VI | S, FS, IS | 0 | 5 (110), | 5 (42†) |
| | D, FD, ID | 5 | 10 (67), | 5 (42†) |

[a] Inoculations with six pairs of strains (one set) were made at separate locations up each tree side from approximately 85 to 190 cm above the ground. Each set was replicated three times except for set III, which was replicated twice.

[b] Treatments: S = two normal strains of the same v-c type; FS = like S, but one strain containing French hypovirulence (H) agents; IS = like S, but one strain containing Italian H agents; D = two normal strains of different v-c type; FD = like D, but one strain containing French H agents; and ID = D, but one strain containing Italian H agents.

[c] Daggers identify the strains that carried French or Italian H agents in treatments FS, IS, FD, or ID.

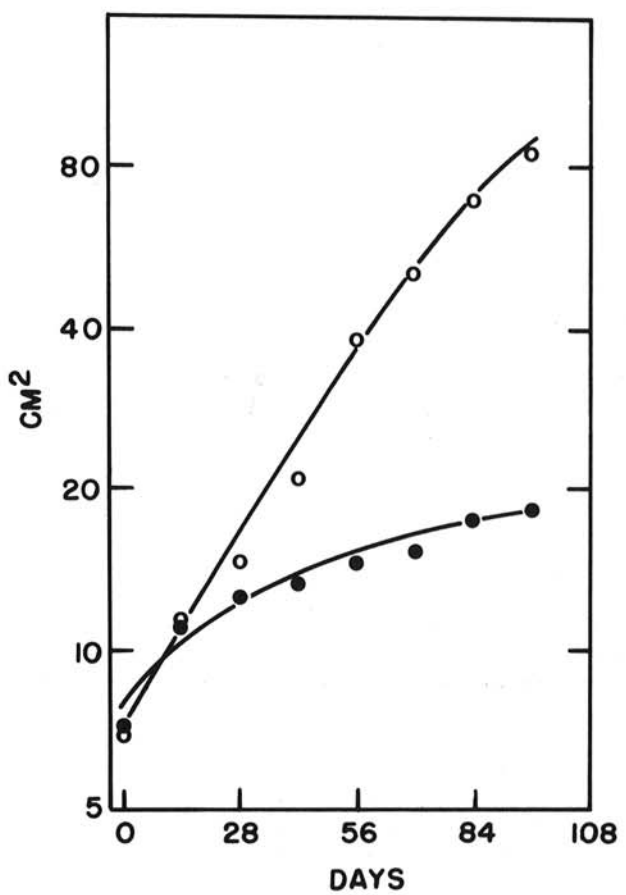


**Fig. 1.** The effect of hypovirulence (H) on the rate of expansion of cankers caused by *Endothia parasitica* on American chestnut trees inoculated with pairs of strains in the same vegetative compatibility (v-c) group. The left strains of each pair were inoculated on 19 May (all normal strains). The right strains of each pair, normal or H, were inoculated on 2 June. Days are counted from 2 June 1980. The area in the absence of H (o) is the mean of 18 cankers (treatment S [see text] in sets I through VI and three replicates). The area with H (•) is the mean of 36 cankers (treatments FS and IS in sets I through VI and three replicates). Mean canker areas are plotted on a log scale.

culture grown on Difco potato-dextrose agar supplemented with methionine (100 mg/L) and biotin (1 mg/L). The inoculum was pressed into a 10-mm-diameter hole cut through the bark with a cork borer. On 2 June a second hole was cut to the right of, and just touching the first, and a plug of the second strain was pressed into it. To prevent desiccation the inoculations were covered with masking tape for 2 wk.

On 2 June 1980, and every 2 wk until 8 September 1980, the lengths and widths of the cankers were measured to the nearest millimeter. These dimensions were converted into areas by assuming they were the axes of ellipses.

## RESULTS

Between 19 May when the trees were inoculated into holes of 78-$mm^2$ area and the second inoculation on 2 June, the canker areas had expanded to a mean of 700 $mm^2$. After 98 days more, a few of the cankers had girdled the trees, the mean area of cankers free of H was 95 $cm^2$, and observations were concluded.

The mean square for the interaction of the six sets by the six treatments (S, FS, IS, D, FD, and ID) by the three replicates was calculated. The square root of this mean square, a standard deviation, was clearly proportional to the mean area. Therefore, the areas were transformed into their logarithms for all further examination.

The decrease in the rate of canker expansion caused by hypovirulence when the two inocula, one free of H agents and one carrying them, are of the same v-c group is evident in Fig. 1. The figure compares the 18 cankers that developed from pairs of inocula of the same v-c group with the 36 that were inoculated with the same strains, but with one of each pair infected with either French or Italian H agents.

When the two inocula were of v-c groups that differ at a single v-c gene, hypovirulence in one inoculum decreased canker growth. Fig. 2 compares the six cankers that developed from pairs differing at a single v-c gene with the 12 that were inoculated with the same strains, but with one of each pair infected with French or Italian H agents.

If the two inocula were of v-c groups that differed at five or more v-c genes, H in one inoculum *scarcely* decreased the rate of canker expansion (Fig. 3). In Fig. 3 as in Figs. 1 and 2, six cankers from normal inocula are compared with 12 in which one inoculum was hypovirulent.

French and Italian H reduced the rate of canker expansion by similar amounts. The effect of each was greater when the two inocula were in the same v-c group (Fig. 4), than when the inocula were in different v-c groups (Fig. 5).

The effect of inocula in different v-c groups in the absence of H is shown in Fig. 6. The six cankers produced by pairs of strains from v-c group 5 or pairs from v-c group 10 are compared to the six cankers produced by pairs composed of v-c groups 5 and 10. These groups differed at five or more v-c genes. After a slow start, the mixed pairs grew somewhat more rapidly than the pairs from the same group.

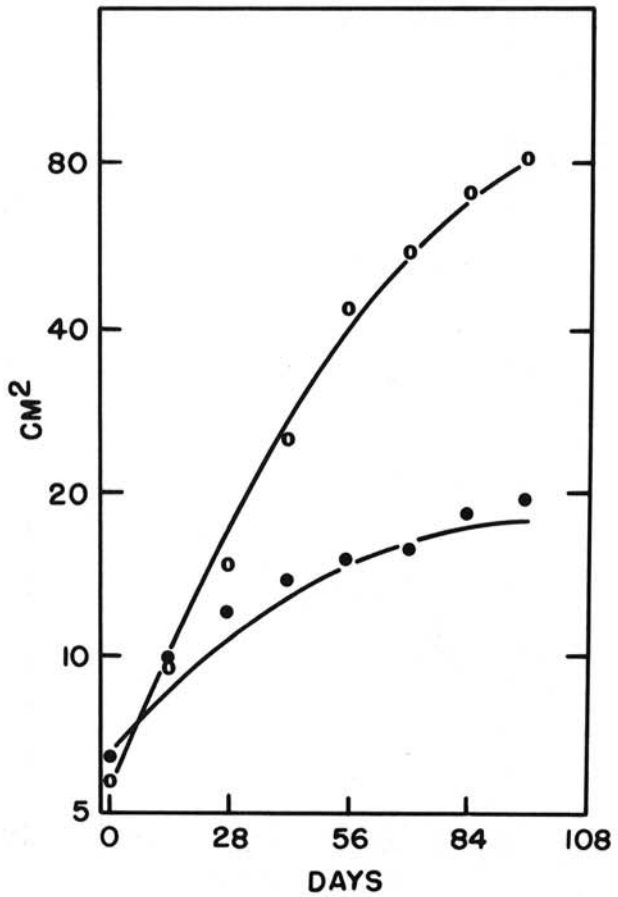


**Fig. 2.** The effect of hypovirulence (H) on the rate of expansion of cankers of *Endothia parasitica* on American chestnut trees inoculated with pairs of strains in vegetative compatibility (v-c) groups determined by different alleles at a single gene. The left strains of each pair were inoculated on 19 May (all normal strains). The right strains of each pair, normal or H, were inoculated on 2 June. Days are counted from 2 June 1980. The area in the absence of H (o) is the mean of six cankers (treatment D [see text] in sets I and II and three replicates). The area with H (•) is the mean of 12 cankers (treatments FD and ID in sets I and II and three replicates). Mean canker areas are plotted on a log scale.

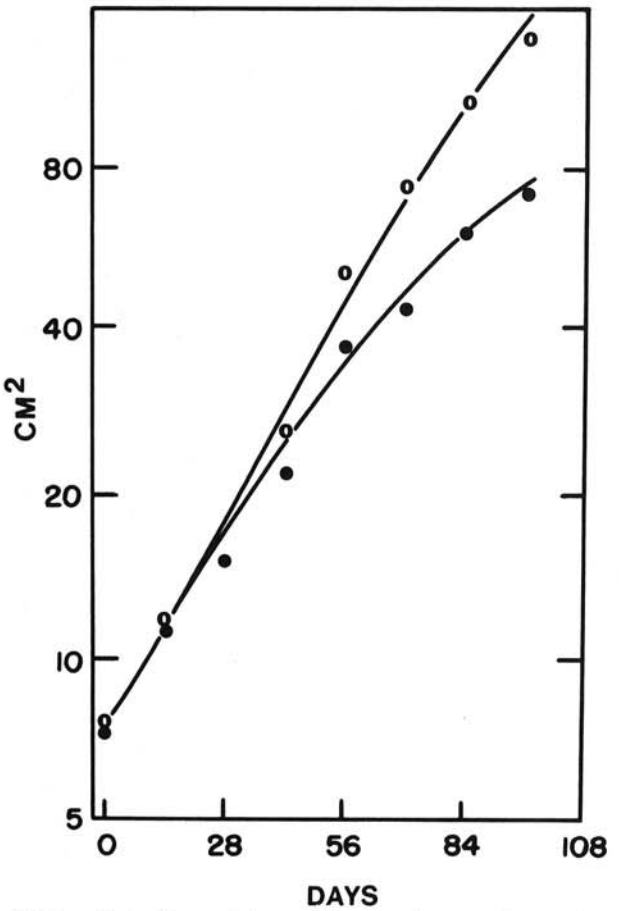


**Fig. 3.** The effect of hypovirulence (H) upon the rate of expansion of cankers of *Endothia parasitica* on American chestnut trees inoculated with pairs of strains in vegetative compatibility (v-c) groups differing at five or more genes. The left strains of each pair were inoculated on 19 May (all normal strains). The right strains of each pair, normal or H, were inoculated on 2 June. Days are counted from 2 June 1980. The area in the absence of H (o) is the mean of six cankers (treatment D [see text] in sets V and VI and three replicates). The area with H (•) is the mean of 12 cankers (treatments FD and ID in sets V and VI and three replicates). Mean canker areas are plotted on a log scale.

The figures mentioned above show curves calculated from orthogonal polynomial equations of the type:

$$y = b_0 + b_1 (x-\bar{x}) + b_2 (x-\bar{x})^2$$

in which $y$ is the logarithm of area, $x$ is days and $\bar{x}$ is the mean number of days after 2 June. Because the predicted responses are computed with orthogonal polynomials, $b_1$ and $b_2$ are mutually independent. The $b_1$ and $b_2$ fit to each of the 108 cankers summarize the $108 \times 8$ observations in $108 \times 2$ parameters, and $b_1$ and $b_2$ are used for subsequent analysis. The units of $b_1$ are a proportional increase per day.

The mean slope or rate $b_1$ of the increase in the area of the cankers where the inocula were in the same v-c group (treatment S) was 0.026 per day and the variation among the means in the first line of Table 2 was insignificant. In other words, the area expanded 2.6% per day, compounded. H (treatments FS and IS) decreased this slope of increase to a third, 0.008 per day (fourth line of Table 2).

When the inocula were in v-c groups differing at one v-c gene, the decrease in the rate of canker expansion was significant and to less than half (line 5 vs line 8, first data column in Table 2). When they differed at two v-c genes, the decrease was significant and to two-thirds. When they differed at five or more v-c genes, the decrease was to three-quarters, but was not statistically significant.

The French H agents decreased the slope of canker expansion more, on the average, than did the Italian. The difference, however, was not statistically significant.

When H was absent, inocula of different v-c groups consistently caused a greater slope $b_1$ than when inocula were in the same v-c group; growth was slow at first and then was rapid (Fig. 6). The difference between treatments S and D was, however, not statistically significant (lines 1 and 5, Table 2).

The curvature $b_2$ of the increase in area was analyzed in the same manner as was $b_1$. No consistent patterns were found in its variation.

## DISCUSSION

In our early work with H in *E. parasitica* we examined mostly strains containing the French H agents. Dodds (7) concluded that the physical-chemical properties of the French H agents are not typical of H agents in strains recovered from superficial cankers in Italy. We have shown here, however, that in the same genetic backgrounds there is little difference between the ability of the French and Italian H agents to arrest canker development either in vegetatively compatible or incompatible strain combinations.

The barrage zone between normal vegetatively incompatible strains of *E. parasitica* on agar media is conspicuous. Assuming that this interaction kills cells in all cases (as it does in *Neurospora* [8]), we expected that pairs of normal strains in different v-c groups would produce smaller cankers than the same strains paired within a v-c group. This seemed to be the case in the report by Jaynes and Elliston (Fig. 2 in [9]) where three normal strains, all in different v-c groups, caused larger cankers alone than when mixed with each other. Nothing is known about the degree of genetic similarity between those three strains. As noted above, the difference in rate of canker expansion between treatments S and D in our experiment

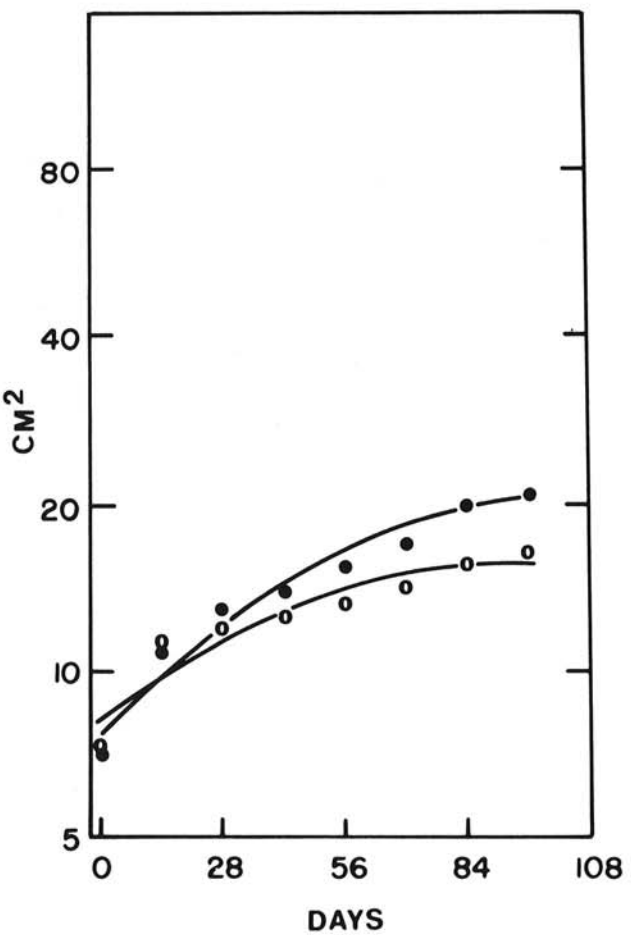


**Fig. 4.** The effects of French (o) and Italian (•) hypovirulence (H) upon the rate of expansion of cankers of *Endothia parasitica* on American chestnut trees when inoculated with pairs of strains in the same vegetative compatibility (v-c) group. The left strains of each pair were inoculated on 19 May (all normal strains). The right strains of each pair, H, were inoculated on 2 June. Days are counted from 2 June 1980. The areas are the means of 18 cankers (treatment FS or IS [see text] in sets I through VI and three replicates). Mean canker areas are plotted on a log scale.

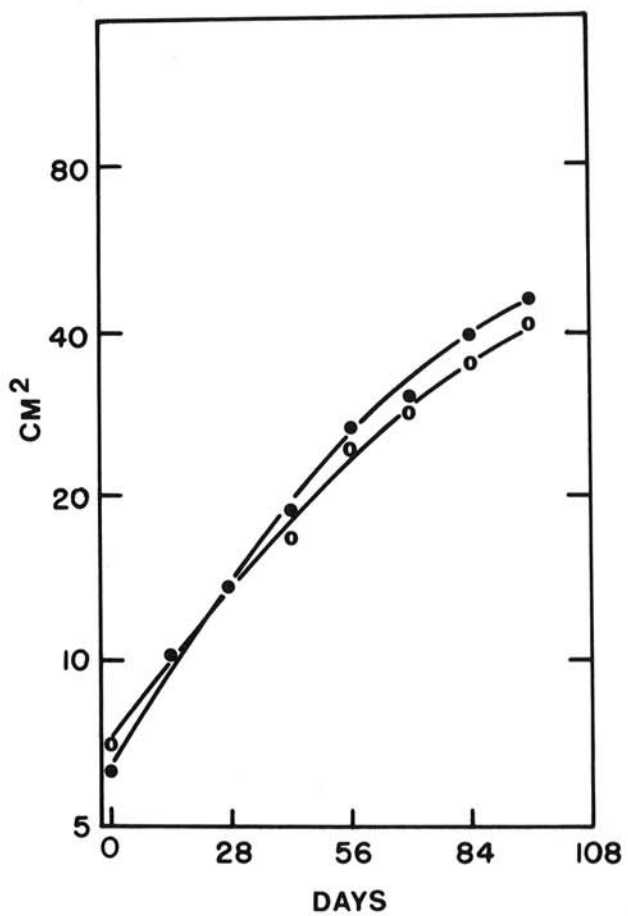


**Fig. 5.** The effects of French (o) and Italian (•) hypovirulence (H) upon the rate of expansion of cankers of *Endothia parasitica* on American chestnut trees inoculated with pairs of strains in vegetative compatibility (v-c) groups differing at 1, 2, or ≥5 v-c genes. The left strains of each pair were inoculated on 19 May (all normal strains). The right strains of each pair, H, were inoculated on 2 June. Days are counted from 2 June 1980. The areas are the means of 18 cankers (treatment FD or ID [see text] in sets I through VI and three replicates). Mean canker areas are plotted on a log scale.

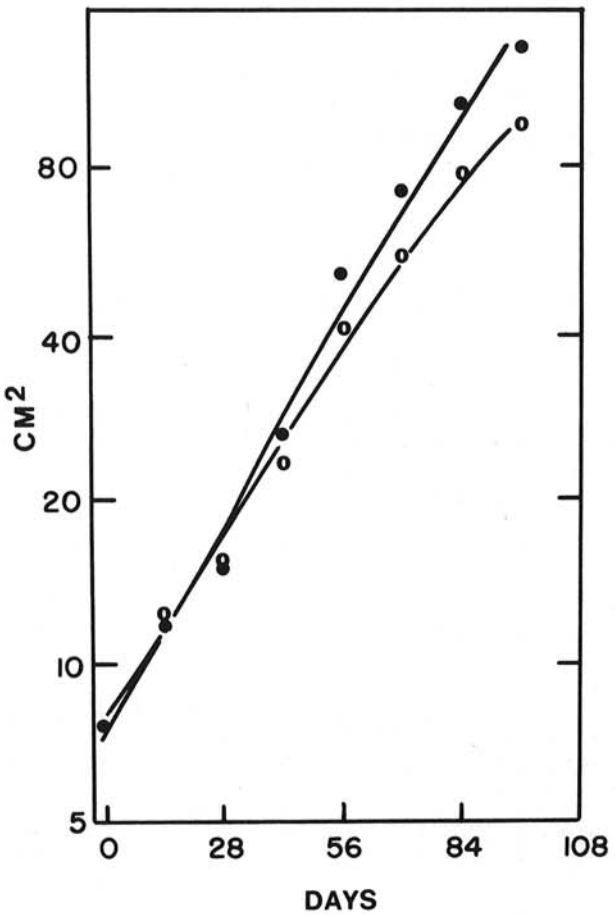


**Fig. 6.** The effect of a difference in vegetative compatibility (v-c) groups upon the rate of expansion of cankers of *Endothia parasitica* on American chestnut trees when hypovirulence (H) is absent. The left strains of each pair were inoculated on 19 May (all normal strains). The right strains of each pair, also normal, were inoculated on 2 June. Days are counted from 2 June 1980. The area with the same v-c groups (o) is the mean of six cankers (treatment S [see text] in sets V and VI and three replicates). The area with different v-c groups (•) is the mean of six cankers (treatment D in sets V and VI and three replicates). Mean canker areas are plotted on a log scale.

was insignificant. Jaynes and Elliston thoroughly mixed the mycelia of their strains, and this close contact of vegetatively incompatible fragments may explain the reduction in growth that they observed. The interaction zone between our paired strains was small compared to their total mixing of mycelium.

Nevertheless, when hypovirulence agents were present in one strain of the pair, the effect of the vegetative incompatibility was clear. We used strains that were crossed in the laboratory and the v-c types among the progeny examined. The number of v-c types present in such progenies gives us a measure of the number of v-c genes segregating in each cross, and thus information on the genetic differences between strains (2). Caten has found that in *Aspergillus* individual *het* genes differ in their strength of inhibition (5) and that the genes have an additive effect in preventing cytoplasmic transfer (4,5). In our experiment with one strain carrying H, canker expansion was significantly limited when the two strains of *E. parasitica* were in the same v-c group and not significantly limited

TABLE 2. Effect of pairing strains of *Endothia parasitica*, with and without hypovirulence (H), on the mean slopes ($b_1$) as a proportional increase per day of cankers on American chestnut trees

| Treatment | Number of v-c genes[a] | | |
|---|---|---|---|
| | 1 | 2 | 5 |
| S[b] | 0.0240(6)[c] | 0.0272(5) | 0.0265(6) |
| FS | 0.0074(6) | 0.0066(5) | 0.0057(6) |
| IS | 0.0096(6) | 0.0115(5) | 0.0085(6) |
| FS and IS | 0.0085(12) | 0.0090(10) | 0.0071(12) |
| D | 0.0280(6) | 0.0336(5) | 0.0308(6) |
| FD | 0.0073(6) | 0.0267(5) | 0.0199(6) |
| ID | 0.0128(6) | 0.0183(5) | 0.0273(6) |
| FD and ID | 0.0100(12) | 0.0225(10) | 0.0236(12) |

[a] The number of vegetative compatibility (v-c) genes is the number of v-c loci with different alleles in treatments D, FD, and ID in the same set of paired strains (see Table 1).

[b] Treatments: S = two normal strains of the same v-c type; FS = like S, but one strain containing French H agents; IS = like S, but one strain containing Italian H agents; D = two normal strains of different v-c type; FD = like D, but one strain containing French H agents; and ID = D, but one strain containing Italian H agents.

[c] The number of cankers used for each mean is shown in parentheses. The error mean square, which is the interaction of treatment by set not caused by number of genes, is $88.6 \times 10^{-6}$ and has 15 degrees of freedom.

when the strains differed at five or more v-c genes. The use of a variety of strains increased the generality of the results. The orderly decrease in effectiveness of H in controlling canker expansion when strains were different at 0, 1, 2, or at least five v-c genes suggest an additive effect similar to that described by Caten.

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