Genetics

Identifying Hypovirulent Isolates of *Cryphonectria parasitica* with Broad Conversion Capacity

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ABSTRACT

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When 118 virulent (V) and 27 hypovirulent (H) isolates of *Cryphonectria parasitica* were paired in culture, 95% of the V isolates were converted to the hypovirulent condition by at least one of the H isolates. The 118 V isolates in 54 vegetative compatibility (v-c) groups included representatives from West Virginia, North Carolina, Virginia, Tennessee, and Italy. The average conversion capacity of the 27 H isolates was 15% and ranged from 0 to 41%. Eight cluster analyses based on different similarity functions were performed to group V isolates according to their susceptibility to conversion. The most useful cluster analysis was based on the square root of the number of sectors converted by individual H isolates and formed nine conversion groups and left only eight isolates ungrouped. Twenty-eight of the 54 v-c groups had more than one V isolate, and isolates in 15 of these v-c groups were also together in conversion groups. Clustering appears useful for determining relatedness among v-c groups. Conidial or mycelial slurries of 7, 15, and 27 H isolates converted 87–93% of 102 randomly selected V isolates from North Carolina. All 102 V isolates were converted by at least one of the H isolate treatments. Conidia were as effective as mycelia in conversion, but more of the colony margin of the V isolates had altered growth from mycelial slurries than from conidial treatments. In a second slurry experiment, minimum numbers of H isolates were selected on the basis of providing maximum conversion of the 118 V isolates in the pairing experiment. Conidial slurries from 4, 7, and 11 H isolates with 82, 91, and 95% conversion of the 118 V isolates in pairings provided conversion of 97, 99, and 85% of the 102 randomly selected, V isolates. Conidial slurries of as few as four H isolates with broad conversion capacity have potential for biological control of chestnut blight on American chestnut because they breach the barrier of vegetative incompatibility.

Additional key words: *Castanea dentata, Endothia parasitica.*

Biological control of chestnut blight with hypovirulent (H) isolates of *Cryphonectria parasitica* (Murr.) Barr (=*Endothia parasitica* (Murr.) P. J. & H. W. Anderson) would be a unique solution to a devastating problem in the eastern United States. Improved survival of chestnut trees in Michigan and Italy is assumed to be the result of a natural buildup of H isolates of the fungus (5,10,19,22). In Connecticut, canker development was slowed for 1–12 mo after cankers were wounded and treated with H isolates (11,12,21). After three growing seasons, however, only 13% of 245 treated cankers were arrested and most trees died of secondary infections during the third and fourth year (7). In the southern Appalachians, 2–4 yr after inoculations with virulent (V) isolates, only 21% of the 220 control trees were alive, whereas 68% of 219 trees that were subsequently treated with H isolates were alive (15). Spraying conidial suspensions of H isolates on

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unwounded cankers enhanced tree survival as much as applying mycelial slurries to wounded cankers (15). Secondary infections by V isolates continued to cause mortality of trees treated with H isolates.

Because the hypovirulence factor is spread to V isolates through hyphal fusion and cytoplasmic exchange between H and V isolates, vegetative compatibility (v-c) and conversion capacity are important considerations. Vegetative compatibility is an indication of the ease with which hyphae of two isolates fuse and exchange cytoplasm. Conversion capacity is the ability of an H isolate to transmit the hypovirulence factor to a V isolate and change the V isolates' growth habit and virulence to the hypovirulent condition. Vegetative incompatibility occurs more commonly among isolates of a fungus than does compatibility, and thus, incompatibility has been seen as a natural barrier to spread of defective, cytoplasmic determinants or viruses (6,9,14). Anagnostakis (1) reported the occurrence of 77 v-c groups among *C. parasitica* isolates tested that would require seven alleles and provide 128 possible v-c groups. Although this large number of v-c groups seemed an insurmountable barrier, Anagnostakis and Day (3) showed that the conversion of V isolates from 38 v-c groups occurred after contact with H isolates from only four v-c groups. Kuhlman and Bhattacharyya (16) found that some native H isolates had the capacity for converting V isolates from 11–13 v-c groups. Even though susceptibility to conversion by indigenous H isolates was common among V isolates recovered along a 300-m study area, H isolates occurred in only four of 41 cankers (16).

Although H isolates do survive in the eastern United States, larger reservoirs of H inoculum may need to be established to provide biological control systems like those in Italy and Michigan (5,10,22). Such biological control systems will need to be established in stands of American chestnut (*Castanea dentata* (Marsh.) Borkh.), where abundant cankers and diverse v-c groups occur. MacDonald et al (18) reported 37 v-c groups were present in 0.16-ha study plots in West Virginia. Nash and Stambaugh (20) reported 34 v-c groups present on American chestnut and 48 v-c groups present on chestnut and oak host species in North Carolina. Besides a diverse pathogen population within areas, individual trees and cankers can contain more than one v-c group (16,17,20).

The purposes of our studies were to determine the breadth of conversion capacity among H isolates towards V isolates from the most common v-c groups in the eastern United States and to use this information so that a conidial suspension from a selection of H isolates would convert ≥90% of V isolates encountered.

MATERIALS AND METHODS

Isolates. Twenty-seven H isolates from the Forestry Sciences Laboratory collection were selected on the basis of conversion characteristics in previously published and preliminary studies (14–16) (Table 1). Some selected H isolates converted V isolates in several v-c groups. If two H isolates converted only V isolates in the same v-c group, the isolate converting more isolates was chosen. Other H isolates were chosen because they represented a variety of v-c groups in the Connecticut v-c system (1).

V isolates from West Virginia were recovered from 880 cankers on American chestnut sprouts in study plots established in areas 10–15 yr after clear-cuts (Table 2). Initially, isolates from 41 cankers were paired in all combinations to identify nine West Virginia v-c groups (17). Two representative isolates from each of the nine groups were selected as testers with subsequent isolates. Additional West Virginia v-c groups were established from merging pairs of isolates that had not merged with the original nine

TABLE 1. Sources of 27 hypovirulent (H) isolates of *Cryphonectria parasitica* and their relative capacity for conversion of 118 virulent (V) isolates

Culture (no.)		Geographic source	Native (N) or induced (I) H[b]	Conn[c] v-c group	Conversion of 118 V isolates (%)
FSL[a]	ATCC				
543	48549	VA	N	(24,34)[d]	41
542	48548	TN	N	(9,36)	31
422	48537	NC	I	(24,34,45)	31
541	48547	TN	N	(24,34)	30
540	48546	TN	N	(16,29,41)	29
128	48540	NC	I	26	26
238	48532	MI	N	9	21
546	48550	VA	N	30	20
514	48539	VA	N	49	17
548	48551	TN	N	(19,42,43)	15
523	48541	Italy	N	8	15
12	48522	France	N	10	14
257	48534	NY	I	(19,42,43, 44,54)	13
370	48535	NC	I	(4,31)	13
527	48544	Italy	N	(30,56)	13
550	48552	TN	N	(19,42,43)	12
434	48538	NC	I	10	12
126	48529	NC	I	-	10
539	48545	Italy	N	50	9
525	48542	Italy	N	40	8
520	48540	Italy	N	12	8
526	48543	Italy	N	(2,30,56)	8
241	48533	France	N	30	8
421	48536	NC	I	30	3
62	48528	NC	I	...	2
549	...	TN	N	(19,42,43)	1
13	48523	France	N	5	0
					Avg. 15

[a]FSL = Forestry Sciences Laboratory collection.
[b]Native isolates recovered from natural infections; induced H isolates resulted from conversion of V isolates in lab or field experiments.
[c]Conn v-c = Connecticut system of vegetative compatibility grouping.
[d]Multiple-merge v-c group; isolate(s) merged with tester isolates from more than one v-c group.

TABLE 2. Relative occurrence in 37 vegetative compatibility (v-c) groups of 880 virulent (V) isolates of *Cryphonectria parasitica* from 880 cankers on American chestnuts in West Virginia

West Virginia v-c group (Conn v-c no.)[a]	V isolates per v-c group (%)
A (9)	14
F (24)	11
B (19), C (15)	7
P (49)	5
D (29), E (30)	4
Q (19, 42, 43, 44, 54),[b] U (37), Delta (5), K (49)	3
Y (36), T, L (2), N (10), X, S (16), Alpha (34, 45), Z (13), 0	2
V (33), H (26), Beta (48), R, Kappa (41), W (50), G (31), Epsilon (49)	1
Eta, Zeta (36)	1
Chi, Theta (7, 54), I (47), J (48), Gamma (30), M (13), Iota (4,31)	>0–0.5
Nonreactive or lost isolates	10

[a]Conn v-c = Connecticut system of vegetative compatibility grouping.
[b]Multiple-merge v-c group; isolate(s) merged with tester isolates from more than one v-c group.

TABLE 3. Relative occurrence in the 28 most common vegetative compatibility (v-c) groups of 330 virulent (V) isolates of *Cryphonectria parasitica* from various hosts in North Carolina

Conn v-c no.[a]	V isolates per v-c group (%)
9	16
43	11
24	8
19	6
15,44	5
8	4
4,34,56	3
26,42,10,41,13,5,36,7,12	2
16,29,33,37,45,40,50,54,55	1

[a]Conn v-c = Connecticut system of vegetative compatibility grouping.

groups. The relationship of most West Virginia v-c groups to groups in the Connecticut v-c system was determined by pairing with Connecticut tester isolates. Some isolates merged with tester isolates from more than one group. These isolates were considered to be in distinct, multiple-merge v-c groups and the Connecticut numbers for the multiple-merge groups were enclosed in parentheses (Table 2).

In North Carolina, 330 isolates were recovered in a systematic survey of v-c types on various hosts in the Piedmont and mountains (20) (Table 3). For v-c identification, all isolates were paired sequentially with groups of tester isolates from Connecticut until a merge reaction was observed. Isolates in multiple-merge v-c groups were common.

Because one objective of our study was to identify H isolates capable of converting V isolates from most v-c groups, we sampled V isolates from a wide range of v-c groups. The 118 V isolates tested for susceptibility to conversion by the 27 H isolates included: one randomly selected isolate from each of the 37 West Virginia v-c groups; one isolate from each of 28 Connecticut v-c groups representing 93% of the North Carolina sample; 32 isolates from Virginia, Tennessee, and Italy, many of which were not converted in a previous study (16); and 21 West Virginia isolates selected from 10 v-c groups to determine variation in conversion among isolates within v-c groups. The v-c testing among the 118 isolates indicated 54 v-c groups were present, including 34 single and 11 multiple-merge Connecticut groups, and 5 West Virginia and 4 Kuhlman-Bhattacharyya (16) v-c groups that were not identified in the Connecticut system.

Conversion by pairing H and V isolates. Two agar disks containing mycelia from each V isolate were paired with two similar disks from each H isolate on PDAmb (Difco potato-dextrose agar with 100 mg of methionine and 1 mg of biotin per liter) (15). Four disks from each V isolate were paired for comparison of the V growth habit with that of the V and H pairs. Direct lighting did not appear critical so stacks of up to six inoculated plates were incubated at 25 C with a 16-hr photoperiod. Changes in growth habit of V isolates caused by conversion were recorded after 4, 6, 8, and 11 days of incubation. In culture, V isolates usually have a fast growth rate, a dense, bright orange, aerial mycelium, and frequent pycnidial production. H isolates have one or more of the following: a slower growth rate, a submerged mycelium that is either white or light orange, and infrequent pycnidial production. The number of contiguous sectors or lines of contact of V with H (zero to four) showing conversion was noted after 11 days. All apparently converted isolates (probable H) were subcultured on PDAmb to confirm a changed growth habit. Subcultures from the V selfed were used for comparison. To further confirm that the changed growth habit was caused by an infectious (invasive) factor, 64 converted isolates were paired with the original V isolates.

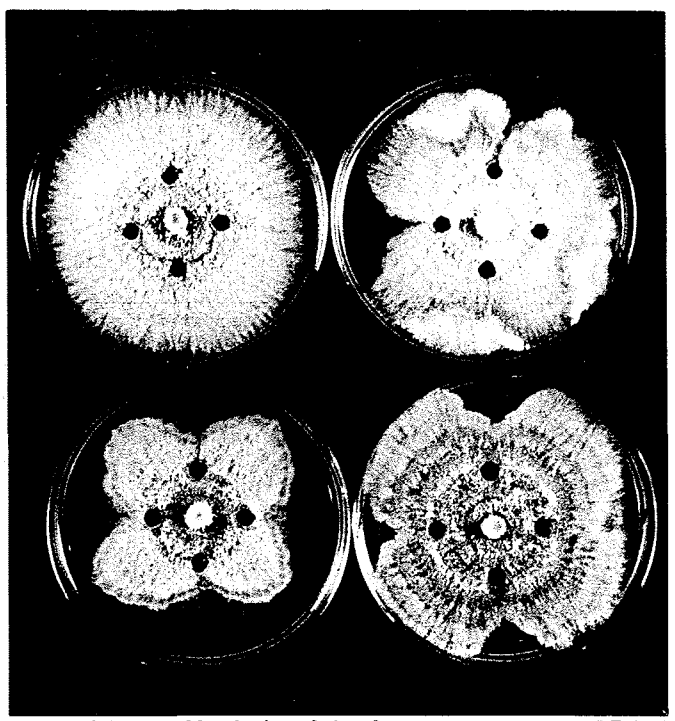

Fig. 1. Eight-day-old colonies of *Cryphonectria parasitica* on PDAmb challenged after 3 days of growth with conidial or mycelial suspensions of H isolates in four wells showing varying responses to the H treatment. (Upper left) no conversion, (upper and lower right) conversion to both fluffy white and submerged growth habit, and (lower left) entire colony margin converted to submerged growth habit.

Conversion groups. Cluster analyses based on different similarity functions were performed to group the isolates (8). Susceptibility to conversion was indicated by speed of symptom development (4, 6, 8, or 11 days) and number of sectors converted (0–4). The 118-row by 27-column matrix A containing data on number of sections converted may be represented by

$$A = \begin{Bmatrix} 0\,1\,0 \ldots\ldots 3 \\ 0\,0(4) \ldots\ldots 0 \\ . \\ . \\ . \\ . \\ 0\,2\,0 \ldots\ldots 2, \end{Bmatrix}$$

where the element in the ith row jth column, $i = 1,2,\ldots,$ 118, and j 1,2,...., 27, indicates the number of sectors of V isolate i converted by H isolate j. For example, the 4 in parentheses means all four sectors of V isolate 2 were converted by H isolate 3. Similarly, the 118-row by 27-column matrix B containing data on conversion day may be represented by

$$B = \begin{Bmatrix} 0\,8\,0 \ldots\ldots 6 \\ 0\,0\,4 \ldots\ldots 0 \\ . \\ . \\ . \\ 0\,6\,0 \ldots\ldots 0, \end{Bmatrix}$$

where element (i,j) indicates the conversion day of V isolate i by H isolate j. A value of zero was assigned when no conversion took place by day 11.

To group the V isolates by cluster analysis, we initially defined similarity (S) between isolates, on the basis of number of sectors converted, as elements of $S_A = AA'$ (where A' is the transpose of A) (8). The similarity between isolates, based on conversion day, was defined analogously by S_B after recording the values of B: 0 day → 0, 4 day → 4, 6 day → 3, 8 day → 2, 11 day → 1 for consistency with the usual notions of similarity and distance. Cluster analyses were performed on the basis of functions of S_A alone, functions of S_B alone, and combinations of S_A and S_B. Because it is not clear just how similarities between isolates should be defined, the original matrix A as well as three alternative transformations of A were used for performing cluster analysis based on conversion. In the first transformation, the elements of A were replaced by their square roots. This transformation kept the elements of the similarity matrix AA′, which consisted of the products of the elements of A, on the same scale as the number of sectors converted. The second transformation recoded nonconversion (zero) as 0 and conversion (1, 2, 3, and 4) as 1, thus disregarding strength of conversion. The third transformation recoded 0, 1 as 0 and 2, 3, 4 as 1.

Conversion with conidial and mycelial slurries. A random-numbers table was used to select 102 of 332 V isolates from the systematic survey of North Carolina (20). V isolates were grown on PDAmb for 7 days at 25 C. Agar disks 5 mm in diameter containing mycelia were taken from colony margins and used to start six plates of each isolate. Incubation was at 25 C with a 16-hr photoperiod. After 3 days, four 5-mm-diameter agar disks containing mycelia were removed at the colony margin so that the disk contained half

mycelia and half uncolonized agar. The four disks were removed along perpendicular lines extending from the center of the plate, which left four wells equally spaced around the margin of the colony (Fig. 1).

For conidial production for the first slurry experiment, the H isolates were grown on PDAmb for 30 days with temperatures fluctuating from 24 to 30 C and a 16-hr photoperiod. For the second slurry experiment, the H isolates were grown on PDAmb for 1 wk under the same growth conditions. Three 10-mm agar disks containing mycelia from each isolate were ground aseptically in 10 ml of sterile distilled water in an Omni Mixer. One milliliter of ground mycelial slurry was spread on the surface of each of three PDAmb plates as described by Barnett et al (4). Plates were incubated as before for 10 days. Conidia were collected from each isolate in sterile distilled water for each experiment. The presence of viable conidia was verified for all isolates by plating spore suspensions on PDAmb. Mycelial suspensions were made from 13-mm-diameter agar disks containing mycelia ground in an Omni Mixer. All slurries were suspended in melted, cooled agar to provide a 0.75% agar suspension.

Before the cluster analysis, seven and 15 H isolates were subjectively chosen to provide conversion capacity for most V isolates and to ensure conidial production. In this first slurry experiment, the six H treatments consisted of conidial and mycelial suspensions of 7, 15, and 27 H isolates. The seven isolates were 238, 422, 128, 542, 543, 514, and 525. Those seven plus 526, 527, 540, 541, 257, 539, 12, and 434 made up the 15. The 27 H isolates were those listed previously (Table 1). Conversion as evidenced by a change in growth habit was visible 4 or 5 days after the H slurries were added.

In the second slurry experiment, H isolates were selected objectively to provide conversion of a maximum number of the 118 V isolates with a minimum number of H isolates. The sequential maximization method identified sequences of H isolates that converted the greatest number of V isolates. Four objective selections were compared to the subjectively chosen seven H's from the first slurry experiment. Selections were: 1) 543, 128, 542, 514; 2) selection one plus 422, 257, 370; 3) selection two plus 238, 525, 548, 546; and 4) selection two plus 238, 527, 540.

TABLE 4. Distribution of conversion response by pairs of virulent (V) and hypovirulent (H) isolates of *Cryphonectria parasitica* by days to conversion and by number of sectors converted

Days to conversion			Sectors converted		
Conversion day	V + H[a] pairs (no.)	Avg. no. of sectors converted	No. of sectors converted	V + H pairs (no.)	Mean days to conversion
4	144	3.5	0	2,702	...
6	224	2.5	1	151	7.0
8	108	1.5	2	92	6.3
11	8	1.5	3	58	5.7
			4	183	4.9

[a] V + H = virulent isolate plus hypovirulent isolate.

RESULTS

Conversion by pairing H and V isolates. When H and V isolates were paired, 112 of 118 V isolates (95%) were converted to H isolates by at least one of the 27 H isolates. The 118 V isolates were converted by an average of four H isolates; one V isolate was converted by 15 H isolates. No conversion occurred with three of four isolates from West Virginia group 0, one of three isolates from multiple-merge v-c group (9,36), and single isolates from Connecticut v-c groups 8 and 55. Conversion capacity of the H isolates ranged from 0–41% (Table 1). H isolates from the same v-c groups varied in their conversion capacity (eg, H isolates 241, 421, and 546 in v-c group 30; 548, 549, and 550 in v-c group (19, 42, 43), and 541 and 543 in v-c group (24, 34)). Conversion was deemed to have occurred only if subcultures of converted sectors retained the abnormal growth habit when compared with growth of the nonconverted V isolate. When 64 abnormal subcultures were paired with their normal V parents, 95% converted the parent isolate. This further demonstrates that the infectious factor was maintained.

Speed of conversion had a normal distribution with a mode at day 6 (Table 4). In contrast, number of sectors converted had peaks at one and four. This contrasting distribution of conversion day and number of sectors converted resulted in $r = -0.57$ based on the

TABLE 5. Percentage of V isolates of *Cryphonectria parasitica* within nine conversion groups converted by each of 27 H isolates

H isolate		Conversion groups and V isolates[a]								
No.	v-c Group	I (13)	II (4)	III (13)	IV (16)	V (19)	VI (7)	VII (8)	VII (11)	IX (19)
549	(19,42,43)	0	25	0	0	0	0	0	0	0
550	(19,42,43)	72	0	0	0	0	0	25	0	5
548	(29,42,43)	92	0	0	0	0	0	50	0	5
257	(19,42,43,44,54)	100	25	0	0	0	0	13	0	0
370	(4,31)	62	100	0	0	0	14	25	0	0
126	...	0	25	38	13	0	0	38	0	0
62	...	0	0	8	0	0	0	13	0	0
542	(9,36)	8	50	100	88	16	0	75	0	0
238	9	0	0	31	100	5	0	63	0	0
422	(24,34,45)	77	0	31	6	100	29	0	0	5
541	(24,34)	69	0	46	6	74	0	25	0	0
543	(24,34)	77	25	69	13	79	86	38	0	11
540	(16,29,41)	31	0	54	6	47	100	50	0	0
241	30	8	0	15	13	0	0	38	0	5
421	30	15	0	0	0	0	0	25	0	0
546	30	15	25	38	31	11	0	100	0	0
526	(2,30,56)	18	0	8	0	5	43	88	0	0
527	(30,56)	15	50	8	6	0	0	88	0	11
525	40	0	0	0	0	0	14	0	0	42
514	49	0	0	23	6	0	14	13	100	16
523	8	0	0	31	6	0	0	25	36	26
520	12	0	0	8	6	0	0	0	36	21
128	26	15	0	0	6	21	14	13	27	100
12	10	15	0	8	0	5	0	0	9	63
434	10	0	0	0	0	16	0	0	9	53
539	50	0	0	0	6	0	0	0	9	53
13	5	0	0	0	0	0	0	0	0	0

[a] Roman numerals indicate cluster groups of V isolates; numbers in parentheses indicate the number of V isolates within each group.

484 V + H pairs that resulted in V conversion. V isolates converted by day 4 averaged 3.5 sectors converted, whereas those converted at days 6 and 8 had 2.5 and 1.5 sectors converted, respectively.

Conversion groups. Eight cluster analyses with different functions of conversion response created nine to 11 conversion groups of V isolates. In contrast to the minimal variation in number of conversion groups, the number of V isolates that were not grouped varied from seven to 16. The 16 ungrouped isolates occurred when response of zero or one sector converted was expressed as zero.

In the analysis of the square root of number of sectors converted, the number of V isolates in the nine conversion groups varied from four to 19 (Table 5). All V isolates within a group were converted by one H isolate that served as the common link in the group, eg, isolate 257 in conversion group I, 370 in group II, etc. Conversion of V isolates within conversion groups by the H isolates varied considerably. Some V isolates in groups II, VI, and VIII were converted by seven or eight of the H isolates, whereas some group VII isolates were converted by 19 of the 27 H isolates.

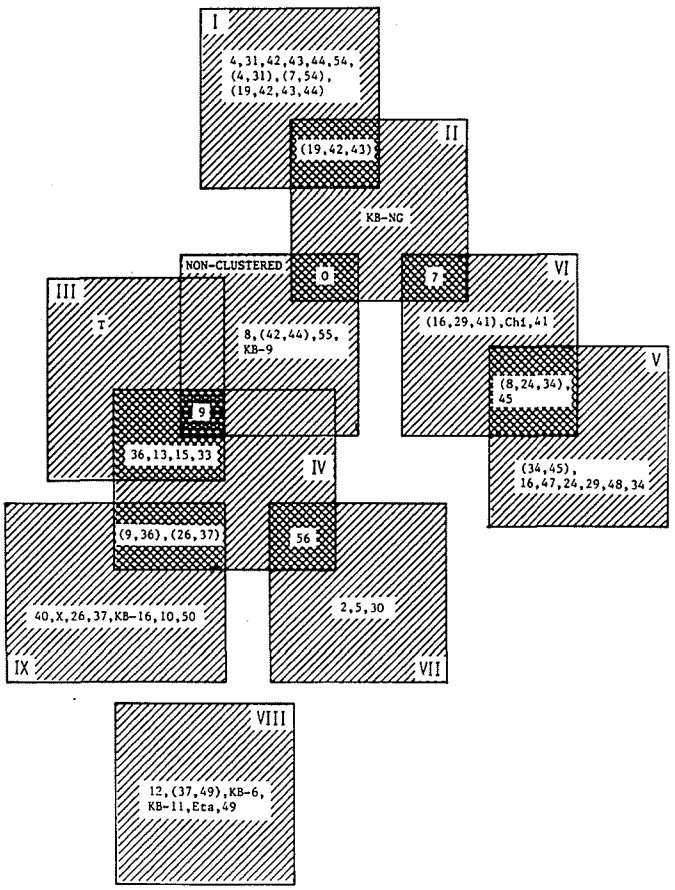

Fig. 2. Diagrammatic illustration of concordancy of vegetative compatibility (v-c) and conversion groups of *Cryphonectria parasitica* as indicated by cluster analysis. The v-c groups are shown as Connecticut numbers, West Virginia letters, and North Carolina (KB-) numbers or nongrouped (NG) isolates. Conversion groups are in roman numerals. Isolates within some v-c groups occurred in two or three conversion groups and are indicated as such by the overlapping rectangles.

Ideally, clustering should group isolates of the same v-c groups in the same conversion groups if the merge and conversion responses are related. We compared 28 v-c groups that had more than one V isolate. The cluster analysis using the square root of the number of segments converted had the best concordance with isolates in each of 15 v-c groups placed in the same conversion groups (Table 6). The other cluster analyses had concordance of 11–14 groups. Each of six isolates from v-c groups 49 and 50 were clustered in conversion group VIII and IX, respectively. Isolates in several other v-c groups had high concordance too, eg, 2, 10, 24, 29, and 48. In addition to v-c groups with multiple isolates, single isolates from several v-c groups were often clustered in the same conversion group in each of the analyses. For example, V isolates from v-c groups 42, 43, 44, 54, Q, and (4,31) were in the same cluster in each of the eight analyses. Conversion groups III and IV shared isolates from five v-c groups and the common link H isolates 238 and 542 appeared to be from closely related v-c groups 9 and (9,36) (Tables 5 and 6). Relationships among conversion groups and v-c groups are illustrated as interlocking groups in Fig. 2.

Conidial and mycelial slurries. In the first slurry experiment, 87–93% of the 102 V isolates from 40 v-c groups were converted in the six treatments (Table 7). All V isolates were converted by at least one of the six H treatments. Either a condial or mycelial H slurry treatment was equally effective in converting the V isolates. Conversion by conidia and/or mycelia averaged 96% within the three H treatments.

The average colony circumference of the V isolates at 3 days when the H treatments were added was 88 mm. Therefore, the H treatment wells eliminated 23% of the circumference because the H isolates grew out from the wells. We estimated 49 and 60% of the expanding margin of the V culture were converted by the conidial and mycelial treatments, respectively.

In the second slurry experiment, conidia of four and seven H isolates converted 97 and 99% of the randomly selected V isolates (Table 8). Increasing the number of H isolates to 10 or 11 decreased the conversion rate to 85 and 86%.

DISCUSSION

The conversion of 95% of the 118 V isolates in pairings with 27 H isolates within 11 days or less indicates compatibility is not a major barrier to spread of the hypovirulence factor. Previously, Kuhlman and Bhattacharyya (16) indicated conversion susceptibility was common over the length of a 300-m study area but the hypovirulence factor had failed to spread. Because the V population in the present study represented 54 v-c groups including 37 from West Virginia, the most common 28 v-c groups in North Carolina and 18 v-c groups from Virginia, these data should be

TABLE 6. Concordancy of vegetative compatibility (v-c) and conversion groups of *Cryphonectria parasitica* as indicated by cluster analysis

Conversion group	v-c Group(s) with indicated number of V isolates					
	One	Two	Three	Four	Five	Six
I	4,(4,31),(7,54),Q,[a]31,42,43,44,54	19,(19,42,43)[b]	...	...	...	...
II	7,[b](19,42,43),[b]KB-NG,[c]0[b]	...	...	...	...	...
III	9,[b]36,[b]T	...	13,[b]33[b]	15[b]	...	...
IV	R,15,[b](26,37),[b]33,[b]56[b]	9,[b](9,36)[b]	13[b]	36[b]	...	...
V	(8,24,34),[b]34,(34,45),45,[b]47	16	...	24,29,48	...	...
VI	7,[b](8,24,34),[b](16,29,41),45,[b]Chi	41	...	...	...	...
VII	56[b]	5,30	2	...	...	...
VIII	12,(37,49),KB-6,KB-11,Eta	...	...	...	...	49
IX	(9,36),[b](26,37),[b]40,X	26,37,KB-16	10	...	...	50
Nonclustered	8,9,[b](42,44),55,KB-9	...	0[b]	...	...	...

[a]West Virginia v-c group Q merged with Connecticut v-c groups 19, 42, 43, 44, and 54.
[b]V isolates from same v-c group in more than one cluster group.
[c]KB = v-c group(s) or nongrouped (NG) isolates of Kuhlman and Bhattacharyya (16).

widely applicable. Our primary objective of identifying H isolates that would rapidly convert a wide variety of V isolates to the H condition was accomplished. Replicating H + V pairs, as Anagnostakis and Day (3) did, might have demonstrated conversion of even more V isolates. However, both the 95% conversion rate and the objective of rapid conversion eliminated the need for replications.

The 27 H isolates ranged from 0–41% in their conversion capacity (Table 1). The average conversion capacity of 15% is less than previous reports of 20–34% (3,10,13). However, the breadth of the V population and the single, brief pairing reduced the probability for conversion in this experiment. H isolates from the same v-c groups varied in conversion capacity even when they came from the same geographic sources (eg, 548, 549, and 550 from Tennessee (Tables 1 and 5). Cytoplasmic factors may be the source of this variation and offer research possibilities. H isolates 543 and 514 were previously identified as having broad capacity for conversion (16). In this study, isolate 543 converted most of the V isolates in four conversion groups, whereas 514 converted most isolates in group VIII only (Table 5). Groups VIII and IX isolates were converted regularly only by H isolates 514 and 128, respectively, whereas several H isolates converted a high percentage of the V isolates in several groups (especially groups I, VI, and VII).

Conversion groups. Kuhlman and Bhattacharyya (16) used a cluster analysis to form v-c groups but had only the presence or absence of the merge response (0 or 1) for the analysis. In this study, time and quantity responses were used and eight cluster analyses were performed: four based on functions of A and S_A alone, two based on functions of B and S_B alone, and two on combinations of A and B. By using the matrices A and S_A, we implicitly introduced the concept of strength of conversion as measured by the number of sectors converted and the concept of similarity between V isolates as measured by the sum of products of the numbers of sectors converted by the same H isolates. Similar concepts were introduced with matrices B and S_B.

Although each of the eight cluster analyses resulted in somewhat different clusters, all eight gave similar groupings to a large number of V isolates. We presented in detail the results based on square root of the number of sectors converted. The properties desired in a cluster analysis are maximum distances between groups and minimum distances within groups. Because conversion of any V isolate occurred in an average of only 4.1 of 27 confrontations with H isolates, many V isolates were clustered on the basis of one or two conversions.

Conversion groups demonstrate the close relationships among some v-c groups. Isolates in v-c groups 10, 37, 40, 50, and X were in conversion group IX as well as being grouped together in most other analyses (Table 6, Fig. 2). Similarly, grouping of v-c groups occurred in conversion groups I, V, and VIII. Recently, Anagnostakis (2) illustrated relationships among 70 v-c groups on the basis of weak barrage reactions and successful transfer of H factors between isolates in different v-c groups. Several combinations of v-c groups she identified were also related in our study on the basis of multiple-merge v-c groups and clustering in conversion groups. Examples are v-c groups 19, 42, 43, 44, 54, and (19,42,43,44,54); 4, 31, and (4,31); 5, 30, and 56; and 24, 34, 45, 47, and (8,24,34). Our multiple-merge v-c groups were intermediates between two or more Connecticut groups and indicated a continuum rather than distinct groups. Because isolates from a v-c group often merged with related v-c groups, these intermediate isolates often linked several groups that became multiple-merge groups here or caused us to place isolates from more than one West Virginia v-c group in one Connecticut v-c group. Our identification of H isolates with conversion capacity toward broad v-c groups confirmed the interrelationships of v-c groups and also simplified the preparation of H slurries for general canker treatment.

Conversion groups III and IV shared isolates from five v-c groups (Table 6, Fig. 2). This sharing of isolates among related conversion groups contributed to the relatively poor concordance. Also, a few isolates were probably misclassified in one system or another. West Virginia v-c group M had been classified in Connecticut v-c group 50 but the selected isolate varied in conversion response and merge response from other v-c 50 isolates and was reclassified in v-c 13 on the basis of a merge response with an isolate from this group. The merge response can only be evaluated subjectively, whereas conversion can be objectively demonstrated. Unfortunately, both methods produced variable results in spite of rigorous efforts to reduce experimental variation. Different batches of agar from the manufacturer seemed to influence isolate growth and conversion.

H isolates converted V isolates from different conversion groups with varying frequencies even if the H isolates were in the same v-c group (Tables 1 and 5). H isolates 548, 549, and 550, from v-c group (19,42,43), converted varying percentages of isolates in groups I, II, VII, and VIII. H isolates 241, 421, and 546 from v-c group 30 had similar dramatic variation in conversion capacity in six of nine conversion group. Anagnostakis and Day (3) reported similar variations when an isolate with three sources of hypovirulence was paired with V isolates from 49 v-c groups.

Conidial and mycelial slurries. The second objective of our study was to convert ≥90% of a randomly selected V population to the H condition. We compared conversion by conidial and mycelial suspensions as well as various combinations of H isolates to find the best hypovirulent source for conversion of V isolates. In the first slurry experiment, conidia and mycelia were equally effective in conversion (Table 7). Because we plan to spray chestnut trees with slurries, conidial suspensions would be more readily adaptable to this system. H cultures growing on PDAmb at fluctuating high temperatures for 30 days produced conidia; however, uniform conditions that produce larger numbers of conidia would be helpful. The Barnett et al (4) method seemed to enhance spore production, but further refinement is needed so that all isolates produce large numbers of spores.

Experimental variation appears to be the main reason for nonconversion because most V isolates were converted by at least one of the slurry treatments. In v-c group 4, conidia from the seven H isolates converted none of five V isolates, whereas mycelia from the seven H isolates converted two of five V isolates. Conversion in

TABLE 7. Percentage conversion of 102 randomly selected virulent (V) isolates of *Cryphonectria parasitica* from 40 vegetative compatibility (v-c) groups by conidia and/or mycelia of 7, 15, or 27 hypovirulent (H) isolates

H isolates (no.)	Slurry type		
	Conidia	Mycelia	Conidia and/or mycelia
7	87	88	94
15	91	90	97
27	88	93	96
Avg.	89	90	96
Within-slurry treatment	100	99	100

TABLE 8. Conversion of 102 randomly selected virulent (V) isolates of *Cryphonectria parasitica* from 40 vegetative compatibility (v-c) groups by conidia of hypovirulent (H) isolates[a]

Treatment (no.)	H isolates (no.)	Conversion of 118 V isolates in pairs (%)	Conversion of 102 randomly selected V isolates (%)
1	4	82	97
2	7	91	99
3	11	95	85
4	10[b]	92	86
5	7[c]	88	99

[a]The minimum number of H isolates was objectively chosen on the basis of conversion of a maximum number of the 118 V isolates when H and V isolates were paired.

[b]In the conservative cluster analysis using conversion of more than two sectors, these 10 H isolates converted 86% of the 118 V isolates.

[c]Seven H isolates subjectively chosen in the first slurry experiment.

v-c group 4 increased slightly with the 15 H treatments with two of five and three of five isolates converted. All five isolates were converted by both mycelial and conidial treatments with 27 H isolates. Two V isolates in both v-c groups 8 and 55 were converted by the slurries although single isolates from these groups had not been converted in the pairing study. There was no evidence of competitive interaction among H isolates to reduce the effectiveness of the slurries with 15 and 27 isolates.

For the second slurry experiment, minimum combinations of H isolates were chosen objectively and sequentially to convert >80, >90, and the maximum possible 95% of V isolates in pairs. According to the sequential maximization method, the 7, 15, and 27 H isolates in the first experiment had 88, 92, and 95% conversion in pairs.

The conversion of 97% of the 102 randomly selected V isolates by spores of four H isolates demonstrates the effectiveness of this research in identifying H isolates of broad conversion capacity. Although 40 v-c groups were represented in the random selection, we were able to choose four H isolates that caused rapid conversion of the V isolates in culture. The reduction in conversion by slurries with 10 and 11 H isolates was apparently due to the dilution of the spore concentration of the four H isolates, because a spore suspension of these four isolates was divided equally among the five treatments in the second slurry experiment.

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