Effects of Hypovirulence in *Cryphonectria parasitica* and of Secondary Blight Infections on Dieback of American Chestnut Trees

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ABSTRACT

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Compatible hypovirulent (H) isolates of *Cryphonectria parasitica* applied to either wounded or nonwounded chestnut blight cankers reduced dieback of American chestnut trees 27–51 mo after inoculation in comparison to dieback of wounded or nonwounded controls. Most control trees were killed back to the point of inoculation with virulent (V) isolates within 15–19 mo after inoculation. Initially, H treatments of wounded cankers promoted healing at the inoculation point, but subsequently the treatments sometimes failed to stop girdling by naturally developing basal cankers or secondary infections by *C. parasitica* at the inoculation point. Thirty-seven months after initial treatment, H isolates were recovered from a higher percentage of live treated trees than dead treated trees and were not recovered from dead control trees. In a second experiment, H isolates were recovered more frequently from nonwounded treated cankers than from wounded treated cankers. Secondary infection of cankers was demonstrated by recovery of V isolates of *C. parasitica* that differed from V isolates used for inoculum in vegetative compatibility (v-c) and/or in conversion susceptibility.

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

Cryphonectria parasitica (Murr.) Barr [= *Endothia parasitica* (Murr.) P. J. and H. W. Anderson] is a highly virulent pathogen that devastated the American chestnut (*Castanea dentata* (Marsh) Bork.) population within the natural range of the host species. Cankers caused by the fungus girdle the host, which causes dieback distal to the infected tissue. Sprouts usually develop below the canker, enabling the root system to survive. Subjective observations by European workers (8,15,17) formed the basis for the hypotheses that natural intensification of hypovirulent (H) factors in *C. parasitica* in Italy was responsible for increased survival of blight-infected European chestnut (*C. sativa*), and that introduction of H isolates in chestnut orchards provided biological control of chestnut blight in France. The former hypothesis can be questioned, however, on the basis of a thorough survey by Palenzona (16) of chestnut forests in the piedmont area near Torino, Italy. A balance was reported between white (apparent H) and normal virulent (V) strains at locations with widespread dieback. In support of the latter hypothesis, Van Alfen et al (18) reported biological control of chestnut blight on American chestnut 42–54 days after inoculation when canker diameters were 38–63% smaller when initiated by V isolates paired with H isolates than in those initiated by pairs of V isolates. For biological control in France, Grente and Berthelay-Sauret (8) wounded cankers before applying the hypovirulent treatment. Jaynes and Elliston (9,10) reported that treatment of wounded cankers with H isolates slowed canker development and reduced tree dieback after two

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growing seasons, compared with nonwounded, V inoculated trees. After three growing seasons, however, most treated trees had dieback because of other cankers (6).

Grente and Berthelay-Sauret (8) called the phenomenon "exclusive hypovirulence," because the H isolates excluded the V isolates during the healing or control process by converting them to H. Conversion of V isolates to H isolates was dependent on the vegetative compatibility (v-c) of the V and H isolates (2,3). Conversion occurred most readily when the isolates were in the same v-c group, but conversion susceptibility was broader than v-c grouping (3,4). Conversion was reported to occur 20–50% of the time between any two V and H isolates (3,8,11,18). When the conservative 20% conversion was used as the success rate, then 14 randomly selected H isolates were needed to ensure 95% conversion of any V isolate (14). 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 infectious nature of this change in growth habit associated with hypovirulence can be confirmed in culture (3).

The two purposes of these studies were: to compare dieback of American chestnut trees 2–4 yr after inoculation with V isolates and treatment with compatible H isolates or appropriate control treatments, and to determine the occurrence of H and V isolates in trees 3 yr after initial treatment. Earlier results of these studies were presented (13).

MATERIALS AND METHODS

Dieback of American chestnut trees. Field experiments were established in June 1978, 1979, and 1980 on the Coweeta Experimental Forest near Franklin, NC, and in June 1979 on the Glenwood Ranger District near Buchanan, VA, in natural stands of American chestnut growing as understory trees beneath a predominantly oak overstory. These experiments are referred to here as 1978 NC, 1979 NC, 1980 NC, and 1979 VA. In each experiment, American chestnut trees 10–20 cm in circumference and free of chestnut blight symptoms were randomly assigned one of five virulent *C. parasitica* inoculum treatments. Trees within V treatments were randomly assigned to H treatments. A cork borer 7 mm in diameter was used to remove a bark disk down to the xylem from the stem of each tree at breast height. Inoculum consisted of a plug of agar and mycelium 7 mm in diameter from 7-day-old cultures of each isolate on Difco potato-dextrose agar (PDA). The inoculum was held in place with masking tape to reduce drying.

Compatible pairs of V and H isolates were randomly selected for each experiment from a collection of 61 pairs (Table 1). All V isolates were recovered from the eastern United States. H isolates had been produced by pairing the V isolates with European H isolates in American chestnut trees and recovering converted American H isolates (11).

In the 1978 NC experiment, each of the five V inoculum treatments included 16 saplings. Six weeks after inoculation, half of the developing cankers were wounded by cutting through the bark with an ax in several places across the canker into healthy tissue. All cankers were then painted with an agar slurry (control) or with an agar slurry of a compatible H isolate (Table 1). Cankers on all nongirdled trees were wounded and treated again in May 1979. These nongirdled trees had all been treated with H isolates in 1978. Different H isolates were used in some treatments in 1979 to enhance conversion.

In the 1979 NC experiment, H treatments were applied to ax wounds across the canker into healthy tissues 1.5, 10, and 24 mo after June 1979 inoculations. Each H treatment was applied to 25 trees (five per V isolate). The four H treatments included an agar slurry control, a compatible H isolate for each V isolate, the five compatible H isolates in a slurry, and a random selection of 28 H isolates in a slurry.

In the 1979 VA and 1980 NC experiments, the wounded treatments were made by removing bark plugs 4 mm in diameter at 2-cm intervals around the canker margin. Agar plugs with and without compatible H isolate mycelium were inserted in the wounds. Nonwounded treatments were applied directly to the canker as a conidial suspension or water spray control in 1979 VA and as a mycelial/conidial, agar slurry, or an agar slurry control in 1980 NC. Treatments were applied 1.5, 10, and 24 mo after inoculation to 25 trees per treatment in 1979 VA and 1.5 and 10 mo after inoculation to 40 trees per treatment in 1980 NC.

Each fall and spring, observations on canker growth, *C. parasitica* sporulation, development of other cankers, and tree dieback were recorded. Inoculations were judged to be healed (callused) if callus formed in the inoculation hole, no signs of *C. parasitica* were present at the inoculation point, and no symptoms of infection, such as orange or sunken bark, callus, or sprouting occurred.

Occurrence of H and V isolates. In September 1981, four bark or wood disks 4 mm in diameter were removed with a flame-sterilized increment hammer from the upper, lower, and two lateral margins of each canker in experiment 1978 NC. Similar sampling was used on all H treated cankers in experiment 1979 VA in June 1982. Disks were surface sterilized for 2 min in 70% ethyl alcohol and plated on PDA. Plates were incubated at room temperature (23–25 C) for 4–7 days. Isolates of *C. parasitica* growing from the disks were subcultured on PDA with 100 mg of methionine per liter and 1 mg of biotin (PDAmb) per liter (2) to subjectively separate V and probable H isolates.

Grouping by v-c. Virulent isolates of *C. parasitica* recovered from 1978 NC in the 1981 sampling were tested for v-c within each V inoculum treatment. Anagnostakis' (2) technique was used, except 10 plugs 5 mm in diameter were used per plate, and incubation was at 28 C in the dark for 7 days followed by 7-day incubation at room temperature (22–25 C) with a 16-hr day length (fluorescent light). All V isolates within an inoculum treatment were paired with all other isolates from that treatment and the V inoculum isolate. Pairs of isolates that merged were placed in the same v-c group, whereas those that formed lines of pycnidia (barrage) were placed in different v-c groups.

Conversion susceptibility. Probable H isolates recovered in 1981 and 1982 from experiment 1978 NC and 1979 VA were paired with their inoculum V isolate in a modification of two techniques (3,7). Isolates were grown on PDAmb for 6 days at 25 C with a 16-hr day length provided by cool-white fluorescent lights. Five plugs of agar and mycelium 5 mm in diameter were taken from the margin of the cultures. Pairs of disks of a probable H and its inoculum V isolate were placed on opposite corners of an 8-mm square in the center of

TABLE 1. Sources of virulent (V) and hypovirulent (H) isolates of *Cryphonectria parasitica* used to inoculate and treat American chestnut trees

Virulent isolate		Hypovirulent isolate		
FSL no.	Source	FSL no.	V+H source[a]	Experiment
26	Franklin, NC	126	26 + Ep 52	1978 NC
		361	26 + Ep 4-6-7	1978 NC
		381	26 + Ep 4-6-7	1980 NC
27	Franklin, NC	62	27 + Ep 54	1978 NC, 1979 NC
		377	27 + Ep 54	1980 NC
28	Franklin, NC	438	28 + Ep 53	1979 NC, 1980 NC
30	Franklin, NC	115[b]	60 + Ep 54	1978 NC
		393	30 + Ep 3	1978 NC, 1980 NC
33	Linville, NC	128	33 + Ep 43	1978 NC, 1979 VA, 1980 NC
60	Mt. Kisco, NY	Ep 3	···	1978 NC
		389	60 + Ep 53	1978 NC, 1979 NC
		392	60 + Ep 53	1979 VA
95	Franklin, NC	366	27 + Ep 4-6-7	1979 NC
209	Andrews, NC	436	201 + 109	1979 VA
220	Andrews, NC	426	214 + Ep 4-6-7	1979 NC, 1979 VA
221	Andrews, NC	421	217 + 62	1979 VA

[a] Hypovirulent isolates were recovered from the V inoculum side of cankers initiated by pairing V and H isolates, eg, 126 from 26 + Ep 52, in an American chestnut sapling. Ep = Connecticut Agric. Exp. Stn. collection.
[b] FSL 115 was not compatible with 30.

the petri dish (Fig. 1). Cultures were incubated at 25 C with a 16-hr day as above. Observation of conversion was made after 4, 6, 8, and 11 days of incubation. Representative converted sectors were subcultured to confirm a changed growth habit before an isolate was designated H.

The conversion relationship of v-c groups within experiment 1978 NC was tested by pairing representative isolates with H isolates used for treatment and 0–4 other H isolates that had converted the inoculum isolate in other experiments (E. G. Kuhlman, *unpublished data*).

V isolates from experiment 1979 VA were paired with H isolates used for treatment to confirm their relationship to the inoculum isolate by conversion susceptibility.

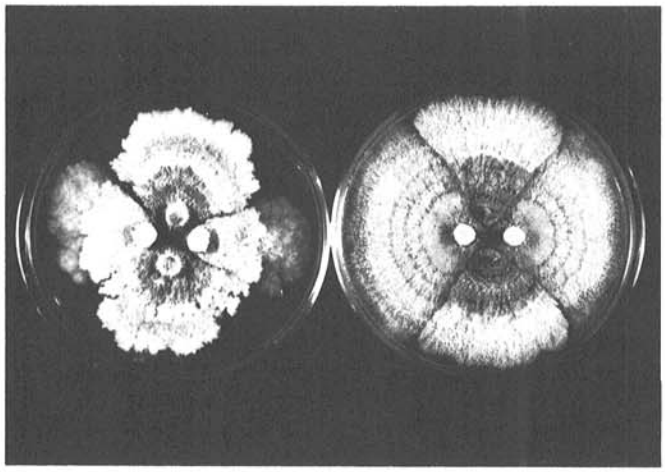

Fig. 1. Conversion capacity of hypovirulent (H) isolates is determined on PDAmb medium along four lines of contact between mycelium of two isolates. In these plates the virulent (V) isolate is the vertical pair and the H isolate is the horizontal pair. On the left, contact of V and H mycelium from pairs of inoculum has resulted in conversion of the V mycelium to the H growth habit along each of the four lines. On the right, the V mycelium has normal growth.

TABLE 2. Effect of wounding and hypovirulent (H) isolates in agar treatments on symptom development after inoculation of American chestnut trees with virulent isolates of *Cryphonectria parasitica*

Experiment	Duration (mo)	Trees having H treatment	Symptoms	Percent affected after H treatment in agar[a] Control	1-H	5-H	28-H
1978 NC	51	40	Dieback	100	75	···	···
			Alive, infected	0	13	···	···
			Alive, callused	0	13	···	···
1979 NC	39	25	Dieback	56	44	36	10
			Alive, infected	44	28	48	86
			Alive, callused	0	28	16	5

[a] H treatments included an agar slurry control, a compatible H isolate for each of five V isolates (1-H), the five H isolates from 1-H in a slurry (5-H), and a random selection of 28 H isolates (28-H) in slurry.

RESULTS

Dieback of American chestnut trees. Trees in experiment 1978 NC treated with H isolates 1.5 and 11 mo after inoculation had less dieback than the agar controls (Fig. 2). However, dieback continued in the treated trees through the fourth year. Only 13% of the treated trees had only callus, a less threatening symptom, after 51 mo (Table 2). In contrast to the dramatic difference in survival in the 1978 NC experiment, the 1979 NC experiment had less dieback in all treatments after 39 mo, although the H treatments had significantly less dieback (Table 2).

Wounding cankers before hypovirulent treatment did not enhance tree survival over that occurring when cankers were not wounded before treatment (Table 3). In experiments 1979 VA and 1980 NC, H treated trees had less dieback than check trees 39 and 27 mo after inoculation, respectively. Live, infected trees were more common in the nonwounded H treatments than in other treatments.

The dieback symptom appeared on trees in control treatments in 14.6, 15.2, and 18.6 mo in experiments 1978 NC, 1979 VA, and 1980 NC but in 29.2 mo in 1979 NC. Dieback appeared on treated trees after an average of 26.4, 24.6, 18.0, and 28.5 mo, respectively.

Basal stem infections by *C. parasitica* have been the cause of dieback of 3 and 28% of 174 control trees and 80 treated trees, respectively.

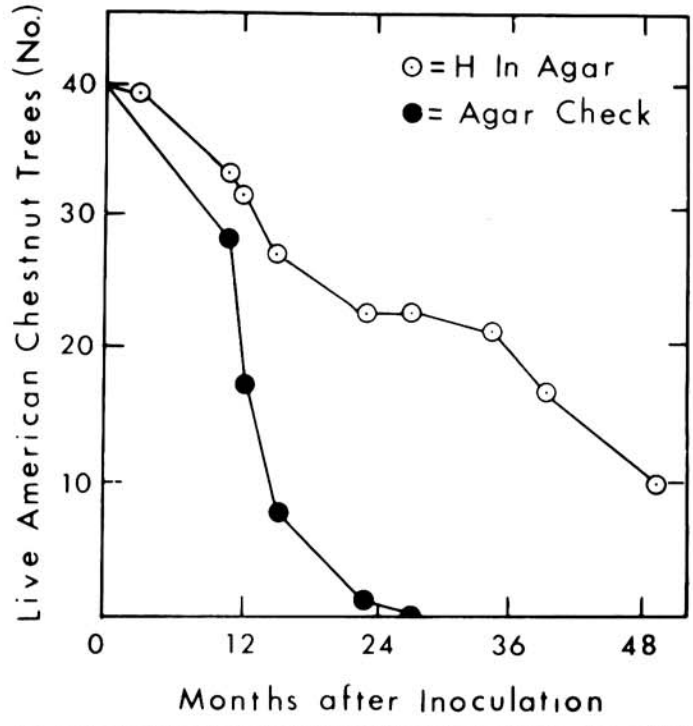

Fig. 2. Survival of American chestnut saplings during the 4 yr after a 1978 inoculation with virulent isolates of *Cryphonectria parasitica* and treatment with compatible hypovirulent (H) isolates or an agar control (check).

TABLE 3. Symptom development on American chestnut 27 and 39 mo after inoculations with virulent isolates of *Cryphonectria parasitica* and subsequent treatment of wounded and nonwounded cankers with hypovirulent (H) isolates

Experiment	Duration (mo.)	Trees having H treatment	Symptoms	Percent affected after treatment: Wounded, agar control	Nonwounded, water control	Wounded, H in agar	Nonwounded, H conidia
1979 VA	39	25	Dieback	92	96	48	32
			Alive, infected	0	0	16	24
			Alive, callused	8	4	36	44
1980 NC	27	40	Dieback	88	90	5	25
			Alive, infected	8	3	41	43
			Alive, callused	5	8	54	33

No effect of V isolate was noted, so no separation of data by isolate treatment was made.

H isolates, v-c groups, and conversion groups in 1978 NC cankers. H isolates were recovered from cankers on 11 of 18 live treated trees and on two of 22 dead treated trees (Table 4). Cankers on check trees yielded no H isolates. The relative recovery of H isolates was greater from live trees than from dead trees.

Pairing for v-c groups among V isolates indicated that all 36 and 25 isolates recovered from cankers initiated by isolates 30 and 60, respectively, were of the same v-c group as the inoculum isolate (Table 5). In contrast, cankers initiated by isolates 33, 26, and 27 had two, four, and seven v-c groups present, including the v-c group of the inoculum isolate, among the 30, 31, and 32 isolates tested. All of the different v-c groups except one were present in treated cankers.

Conversion of V isolates can occur along any of the four lines of contact between the V and H isolates (Fig. 1). Conversion seems to occur along all four lines of contact when the two isolates are closely related and along fewer lines when isolates are of a more distant relationship. The relative ease of conversion of V isolates of the same v-c group as the inoculum isolate was very different from V isolates in other v-c groups (Table 5). For example, V isolates from inoculum treatment 27, v-c group E, were readily converted by H isolates 62 and 436 (100% conversion of all four isolates along the four lines of contact). No conversion occurred when V isolates in this group contacted H isolates 126 and 257 and only 6% (1/16 lines of contact) resulted in conversion by H isolate 421. Isolates in the other six v-c groups from inoculum treatment 27 were not converted by H isolates 62 and 436. Similar differences in conversion susceptibility among V isolates from different v-c groups were observed in isolates from inoculum treatments 26 and 33.

TABLE 4. Relative occurrence of hypovirulent (H) isolates in cankers on live and dead H-treated stems and relative recovery of H and virulent (V) isolates 37 mo after V inoculation and H treatment in 1978 NC

Virulent inoculum isolate	Stems with H isolates present per total stems		Recovery of H isolates as a percentage of *Cryphonectria parasitica* recovered[a]	
	Live	Dead	Live stem	Dead stem
26	2/5	1/3	29	14
27	2/3	1/5	36	13
30	···	0/8	···	0
33	4/7	0/1	50	0
60	3/3	0/5	70	0
Totals	11/18	2/22		
Mean percent			46	5

[a] Four samples were taken from each stem in each category.

Recovery of *C. parasitica* isolates from 1979 VA. Isolates of *C. parasitica* were recovered with equal frequency from cankers that were wounded or nonwounded before compatible H isolates were applied (Table 6). H isolates were more commonly recovered from nonwounded cankers treated with conidia than from wounded cankers treated with mycelium. Most H isolates came from cankers on live trees. V isolates unrelated to inoculum V or H isolates, as indicated by no conversion by the treatment H isolate, were more common in wounded, H mycelium-treated cankers than in nonwounded, H conidia-treated cankers. In the mycelial treatment, unrelated V isolates were more commonly recovered from cankers on dead trees.

DISCUSSION

Treatment of wounded or nonwounded chestnut blight cankers with compatible H isolates reduced dieback of American chestnut trees 27–51 mo after initial inoculations in comparison to dieback of appropriate control treatments. Only 21% of 220 control trees in four experiments had not died back, whereas 68% of 219 H-treated trees had not died back by September 1982. In the 1978 NC experiment, trees with control cankers and cankers treated with an incompatible H isolate (30 by 115) had died back 15 mo after inoculation. The incompatible H isolate was an error in experimental installation. Although some trees with cankers treated with compatible H isolates died back as rapidly as trees with control cankers, generally the H treatments increased the time to dieback as well as enhancing survival. In the 1978 NC experiment, recent dieback was due to renewed activity in the treated cankers rather than to new cankers.

Disease control can have many meanings. Ainsworth (1) defined it as the way "to prevent or retard the development of a disease." Apple (5) suggested that control implies eliminating the disease problem, whereas disease management indicates limiting disease loss below an economic threshold. Much of the recent research on hypovirulence in *C. parasitica* has considered briefly retarded disease development to be synonymous with biological control. Maintenance of a live host population in 1978 NC for more than three times as long as the check population could be considered a control (51 vs 15 mo); however, trees have continued to die back so that disease prevention has not occurred. Further research is needed to determine whether a hypovirulent population of broad conversion capacity can be self-perpetuating and capable of long-term retardation of the disease.

Jaynes and Elliston (9) reported that spraying nonwounded cankers with conidial suspensions from H isolates reduced canker development 4 mo later compared with canker development in untreated checks. Data (Table 3) indicate that treatment of nonwounded cankers with conidia from H isolates was as effective in preventing dieback 2 and 3 yr after inoculation, as was treatment of wounded cankers with mycelial slurries of the H isolates. The

TABLE 5. v-c Group designation for virulent (V) isolates recovered from cankers started by V inoculum isolates. Relative conversion of some V isolates from each v-c group was determined by pairing with hypovirulent (H) isolates from various v-c groups

Virulent inoculum isolate	v-c Group	V isolates (no.) Recovered	V isolates (no.) Paired	Conversion[a] by H isolate and v-c group: 361 A	126 A	62 E	436 P	257 O	421 Q	115 O	247 L	393 L	434 R	128 M
26	A	25	3[b]	100	100									
	B, C	4	3	0	0									
	D	1	1	75	0									
27	E	14	4[b]		0	100	100	0	6					
	F	9	3		33	0	0	0	0					
	G	5	3		0	0	0	58	0					
	H–K	4	4		0	0	0	0	0					
30	L	36	7[b]							0	71	46	68	
33	M	26	3[b]											100
	N	4	2											0
60	O	25	5[b]					90					0	

[a] Conversion is presented as percent of total segments (four segments per V isolated paired) converted to H by each H isolate.
[b] One isolate in this group was the inoculum isolate.

TABLE 6. Effect of canker treatment with mycelium or conidia of hypovirulent (H) isolates on relative recovery of H isolates and virulent (V) isolates related and unrelated to the inoculum 36 mo after inoculation in experiment 1979 VA

Factor	Incidence (%)[a]	
	Wounded, mycelium	Nonwounded, conidia
Cryphonectria parasitica recovered	51	55
Total H isolates recovered	12	36
H isolates from live trees	8	30
Unrelated V isolates recovered	32	5
Unrelated V isolates from live trees	12	3

[a] Based on 100 samples, four from each of 25 trees.

effectiveness of conidia from H isolates applied to nonwounded cankers in preventing dieback is encouraging, because it demonstrates that wounding is not a prerequisite to establishing effective H infections and suggests that natural spread of the H isolates may be possible.

The presence of more H isolates in live treated trees than in dead check or dead treated trees in 1978 NC provides data in support of the suggestion by Grente and Berthelay-Sauret (8) that in France H isolates become dominant in treated trees. Even more impressive is the recovery of more H than V isolates from nonwounded cankers treated with conidia from H isolates in 1979 VA (Table 6). The treatment of young wounded cankers with mycelium of H isolates caused rapid healing with callus. Nonwounded cankers sprayed with H conidia retain signs or symptoms of blight, but the blight does not cause dieback. For long-term maintenance of the American chestnut, the latter type of disease expression may be more desirable if these infections can provide H inoculum for dissemination.

A balance of H and V isolates in nonlethal cankers may also limit infections by unrelated V isolates. Wounded H treated cankers more frequently had unrelated V isolates present than did nonwounded H-treated cankers. Rapid formation of healing callus after wounding and H treatment appears encouraging with regard to control, but the callus may be a prime infection court for V inoculum.

Many problems still need to be solved before hypovirulence can be considered a useful tool for disease management. Maintenance and spread of hypovirulence is a major concern. Since H isolates grow and sporulate at a slower rate than V isolates, they are at a competitive disadvantage. Willey (19) reported spread of hypovirulence among cankers on H-treated trees but presented no evidence for spread to cankers on untreated trees. Previously, Kuhlman (12) indicated the rate of spread of hypovirulence in France (1–2 m/yr) was 16,000 times slower than the rate of spread of the virulent forms in the United States (16–32 km/yr). Offsetting this advantage may be difficult within the natural range of the American chestnut.

Virulent isolates recovered from many cankers were from different v-c groups than the inoculum. These isolates differed both in v-c grouping and in conversion susceptibility in 1978 NC and in conversion susceptibility in 1979 VA. Because the isolates were not converted by the H isolates used for treatment, they may cause dieback of some trees and were probably the cause of dieback of other stems. These isolates probably came from secondary infections of the cankers since the disease is active in the study areas. Elliston (6) reported dieback due to secondary infections of treated trees 3–4 yr after treatment. Establishing an H population that is able to maintain control of secondary infections will require further research. The randomly selected 28 H isolates used in 1979 NC reduced dieback, but maintaining such a large H population is probably not necessary because a random selection of 14 H isolates should convert 95% of the population.

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