Survival of American Chestnut Trees: Evaluation of Blight Resistance and Virulence in *Endothia parasitica*

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ABSTRACT

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Large (22- to 114-cm diameter at breast height [DBH]), surviving American chestnut trees in the natural range were evaluated from 1978 to 1982 for blight resistance and/or the presence of *Endothia parasitica* strains with low virulence. Canker length measurements following inoculation of virulent *E. parasitica* on grafted scions, seedlings, and excised stems indicated that some surviving trees are blight resistant. In situ inoculation trials on large, surviving trees with virulent *E. parasitica* indicated that superficial canker development is also an important characteristic associated with blight resistance in American chestnut. Strains of *E. parasitica* that produced short, superficial cankers on blight-susceptible American chestnut stump sprouts were associated with large, surviving trees, smaller trees (5–14 cm DBH) growing near large trees, and small stump sprouts (4–19 cm DBH). Of 542 isolates tested, 113 (20.8%) had low or intermediate virulence. Eight of eight low-virulence and one of three intermediate isolates tested contained dsRNA. The incidences of low-virulence isolates in the *E. parasitica* populations from large, surviving trees and from small stump-sprout trees in Virginia-West Virginia were 14.9 and 4.3%, respectively, whereas the incidences of intermediate strains were 13.2 and 2.6%, respectively. Many American chestnut trees may survive because of the combined effects of blight resistance and low virulence of the pathogen, but others may survive because of a single factor.

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

The American chestnut [*Castanea dentata* (Marsh.) Borkh.] grows as a small tree (usually <8 cm in diameter) in the understory of forests or in forest clearcuts of the southern Appalachians. Near Blacksburg, VA, American chestnut stump sprouts (>0.8 cm in diameter) may occur at densities greater than 1,000 trees per hectare. Many of the small understory trees that have escaped infection may be >20 yr old (14). In forest clearcuts, most stems are killed by blight in 10 to 12 yr, although stumps of these trees may resprout (14). Blighted, large (>25 cm DBH) American chestnut trees, which survived the initial blight pandemic, grow in the southern Appalachians. Younger (stump-sprout or seedling) large, blighted trees also grow (Fig. 1). Frequencies of large trees range from 0 to 7 per county, and 10–95% of the crowns of these trees are alive. Natural cankers on one large, surviving tree (Fig. 1B) in Virginia expanded little over a 3-yr period (14). Some American chestnut trees might survive because they resist blight sufficiently, because they are infected with strains of the chestnut blight fungus, *Endothia parasitica* (Murr.) P. J. and H. W. Anderson that have low virulence, or because of a combination of these factors.

Previously, we found that some isolates of *E. parasitica* from surviving American chestnut trees in the natural range had low virulence, but most were virulent strains (11,12). Only one or a few *E. parasitica* isolates per surviving tree were used in these studies, and more extensive pathogenicity trials are needed to confirm this finding (12). Elliston et al (8) isolated a low-virulence strain from an American chestnut growing in an area of Michigan outside the natural range of this species. Jaynes and Elliston (16,17) reported an association of low-virulence strains of *E. parasitica* with surviving trees in the natural range. The present study was undertaken to evaluate the roles of blight resistance and the virulence level of *E. parasitica* in the survival of some American chestnut trees. Some information was reported earlier (13,15).

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MATERIALS AND METHODS

Blight-resistance trials. Blight-resistance trials were performed on bark-grafted scions of surviving American chestnut trees, on seedling progeny of surviving trees, directly (in situ) on surviving American chestnut trees, and on excised stems of surviving trees. Two virulent isolates of *E. parasitica* (CR and WK), examined previously (12), were used in the in situ and excised stem trials, and the CR isolate only was used in the trials with bark-grafted scions and seedlings.

Bark grafts of American chestnut scions were made on three Chinese chestnut trees, each about 30 yr old, at Blacksburg. Scions from two blight-susceptible seedlings of an Iowa American chestnut tree served as controls. Inoculations were made in June, 1980, when the bark-grafted scions were 3 yr old (11–35 mm in diameter). Seedlings from surviving American chestnut trees were planted in a field at Blacksburg and were 3–4 yr old (12–25 mm in diameter) when inoculated in June 1980. Seedlings from a blight-susceptible American chestnut tree growing in Wisconsin were used as controls. A nested, completely randomized design was used for both graft and seedling trials.

Large, surviving American chestnut trees were inoculated in situ in 1981 on one small branch or trunk sprout (29- to 40-mm diameter) and on one large (40- to 60-mm diameter) branch of each tree. All trees, located in Virginia, West Virginia, Ohio, and Pennsylvania, were inoculated within a 3-day period in May. Seven American chestnut stump-sprout trees of similar stem size (26–55 mm DBH) growing in the Jefferson National Forest, VA, were inoculated as controls. A systematic, spatial-inoculation design was used for each tree. Excised branch segments (25- to 75-mm-

diameter) of surviving trees were inoculated in 1979 and in 1980 by methods similar to those of Elliston (7) and were incubated at 27–28 C. Excised branches were collected in the dormant season between 1 February and 9 April. Dormant stump-sprout stem sections from the Jefferson National Forest, VA, were used as controls. A completely randomized design (one block) was used.

All inoculations of virulent strains of *E. parasitica* were done by the agar-disk, cork-borer method used previously (12). On bark-grafted scions and seedlings, inoculum disks 1.5 mm in diameter of Difco potato-dextrose agar (PDA) were used, whereas disks 6 mm in diameter were used for excised stems and for the in situ inoculations. Inoculations were 15–30 cm apart. For each treatment, one to five scions, with two CR inoculations per scion, and five seedlings, with two CR inoculations per seedling, were used. In in situ trials, five CR and five WK inoculations were made for each surviving tree, and three CR and three WK inoculations were made on each stump sprout. Six CR and six WK inoculations were made on each excised stem treatment type. Inoculations were covered with masking tape to prevent desiccation. Final canker lengths (found reliable for blight resistance trials [5]), ratings of stromata production, and bark swelling were determined after specified time periods in all trials; ratings of superficiality of bark necrosis (following canker dissection) and staining of bark and wood tissues were determined for the in situ resistance trials. The final determinations were made during a 3-day period in May, and preliminary measurements were made in December. In the bark-grafted scion, seedling, and excised-stem tests, canker lengths were also determined every 2–3 days from 5 to 15 days until 37 or more days after inoculation, to estimate initial lesion size (14,15) and to determine net canker length. All length measurements were made with a millimeter ruler.

Virulence determinations. Pathogenicity trials of *E. parasitica* isolates on American chestnut stump sprouts were used to determine virulence level in all instances from 1979 to 1982. Two surviving American chestnut trees studied previously (12) were sampled extensively for *E. parasitica* isolates. Cankers on all portions of the trees (bole, main, and secondary branches) were sampled. Samples consisted of small bark patches obtained from canker margins with a 15-mm-diameter cork borer or with a knife. They were obtained from continuous, lower cankers to obtain the inner radial margins, and from lateral canker margins of discrete cankers, located higher on the trees. Approximately 100 samples were obtained from the LS-1 tree, and 30 were obtained from the LS-9 tree. Similar procedures were used to sample large, surviving American chestnut trees, small trees growing near surviving trees, and stump sprouts in Virginia, West Virginia, Ohio, and Pennsylvania, except that fewer samples (1 to 17) were obtained per tree.

Virulence trials were performed on stump sprouts growing in the Jefferson National Forest near Blacksburg, VA. Surface-sterilized bark samples from cankers were plated on acidified PDA (APDA), and *E. parasitica* isolates growing from samples were transferred when necessary to PDA plates to obtain axenic cultures. In some instances, they were then transferred to two slants of PDA. Unless otherwise specified, no preference during subculturing was given to *E. parasitica* colonies with abnormal morphology; representative isolates growing from bark samples typically were used in virulence trials. The agar-disk, cork-borer (6-mm-diameter) method of inoculation (12) was used in all tests, in which cultures 7 to 10 days old were used. For each *E. parasitica* isolate, five stump sprouts (25- to 82-mm DBH) were inoculated, and each stump sprout was inoculated with five different isolates of *E. parasitica*. A disconnected Latin square design was used, and inoculation sites were 30–40 cm apart. Stump sprouts used were usually single, with the nearest neighbor at 2–30 m. Inoculations usually were made the last week of May or the first week in June, and canker evaluations (length, stromata production, canker superficiality and swelling, and bark and wood staining) were made in early to mid-October. An intermediate measurement of canker length was made in early to mid-August. Virulence level within each year was determined by considering the mean total canker length, mean net canker length (October measurement minus August measurement), and the superficiality of canker development. Auxiliary information was assays for dsRNA, a probable determinant of cytoplasmic low virulence (22), for 11 isolates. After analysis of variance, canker-length data for each year were subjected to univariate cluster analysis (23) to help determine virulence groups. All years were not analyzed as a group, so that variance would be minimized. Control isolates CR and WK served to make comparisons among years and tree types.

RESULTS

Blight-resistance trials on bark-grafted scions. Linear canker growth (after initial lesion formation [14,15]) due to the virulent CR isolate on bark-grafted scions of the large surviving LS-18 tree began significantly ($P < 0.05$) later than canker growth on scions of the control trees S-A and S-B (Table 1). Also, 45 days after inoculation, mean canker length was significantly less for the LS-18 tree than for the control trees. After 45 days, cankers on scions of the surviving trees LS-13 (Fig. 1B), LS-21, and LS-15, and one small tree (AT-13) adjacent to LS-13, were significantly shorter than cankers on scions of S-A, but not on scions of S-B. The latter had larger cankers than the surviving trees, but fewer replicates. Canker measurements after day 45 were not analyzed because of the death of the stems with the smallest diameters. All the stems distal to cankers on these juvenile stems were eventually killed.

Blight resistance trials on seedlings. Linear canker growth on seedlings from the surviving LS-24 tree began significantly ($P < 0.05$) later than canker growth on control seedlings S (Table 2). Canker lengths 46 days after inoculations were significantly less on the seedlings from the surviving trees LS-24, LS-18, LS-23, and LS-9 than on the control S seedlings. Cankers on the LS-20 seedlings were shorter than on the control seedlings, but the difference was not statistically significant. Canker measurements after 46 days were not analyzed because of the death of the stems having the smallest diameters. All stems distal to cankers on these juvenile stems were eventually killed.

In situ blight resistance trials on surviving trees. One year after inoculation with virulent *E. parasitica*, mean canker length on the surviving LS-10 tree was significantly ($P < 0.05$) less than on the seven stump-sprout (control) trees (Table 3). Mean canker lengths on four other surviving trees (LS-18, LS-14, LS-13, and LS-16) were less than on almost all control trees, but the differences were not statistically significant. The range in mean canker length at the

TABLE 1. Canker growth statistics of the virulent isolate CR of *Endothia parasitica* on bark-grafted scions of large surviving (LS), blight-susceptible (S), or associated tree (AT) American chestnut trees for 1980

Scion code and tree type	Cankers (no.)	Postinoculation canker length (mm)[w] at: 10 days	45 days	Initiation of linear canker growth[w,x] (days)
LS-11[z]	2	10 bc	...[y]	10.0 c
S-A	10	19 a	50 a	12.8 c
LS-20[z]	8	20 a	50 a	12.5 c
S-B	4	11 bc	41 ab	16.8 bc
LS-9[z]	8	19 a	41 ab	16.3 c
LS-15[z]	10	12 b	38 b	13.2 c
LS-21[z]	10	13 b	37 b	16.1 c
LS-13[z]	6	11 bc	32 b	23.2 bc
AT-13[z]	2	18 a	32 b	33.5 b
LS-18[z]	4	4 c	10 c	78.0 a

[w] Means within columns followed by the same letter are not significantly different ($P < 0.05$) by Duncan's multiple range test. There were two inoculations per scion, and one to five scions per treatment.

[x] The time of start of linear canker growth was determined as the time after initial lesion (14,15) formation that the increase in canker length from the previous observation exceeded 2 mm. Observations were 2–3 days apart, starting 5 days after inoculation.

[y] These scions were killed before day 45.

[z] Mean and range of diameter (DBH) of parent trees were 59.4 cm and 41 (except AT-13 = 18 cm) to 114 cm, respectively. Mean and range of crown rating (percent crown alive) were 51% and 20–85%, respectively.

stump-sprout location was greater than among all surviving tree locations. Because the inoculated stems in these tests were generally larger and the bark thicker than stems in the graft or seedling tests, they were dissected to determine the degree to which cankers had reached the vascular cambium. This measurement was combined with the canker length measurement to determine a canker severity index for each tree (Table 3). For example, canker lengths were multiplied by 1.0 when all replicate cankers were superficial, with little or no (0–9%) necrosis at the vascular cambium, and by 10.0 when 90–100% of the canker areas reached the vascular cambium. All cankers on five of seven control stump sprouts extended to the vascular cambium over 90–100% of the canker areas, but eight of 10 large surviving trees had cankers that exhibited various degrees of superficial canker development. Mean canker severity indexes for six large, surviving trees (LS-10, LS-18, LS-14, LS-13, LS-16, and LS-9) were significantly different from mean canker severity indexes of all seven control trees (Table 3). Four trees (LS-10, LS-18, LS-14, and LS-13) had cankers that were almost completely superficial. In instances in which cankers were not completely superficial, small necrotic rays of tissue were observed extending radially toward the xylem at various locations beneath the superficial canker. Superficial cankers were also observed on the large (80.5-cm DBH, and surviving the blight pandemic) LS-17 tree, the probable pollen source of the LS-18 seedlings, but most inoculated branch areas of this tree died by May 1982, due to two natural cankers. Thus, sufficient replicates were not obtained for statistical analyses. There was no correlation between inoculated branch size, within the limits studied, and canker severity index within a tree or among surviving trees. Swelling of cankers was usually associated with short, superficial cankers. All stems of stump-sprout trees, and five of six branches on the LS-19, LS-12, and LS-1 trees died within 1 yr. Almost all branches (12 of 14) of the LS-15, LS-9, LS-16, LS-13, LS-14, LS-18, and LS-10 trees did not die within 1 yr.

TABLE 2. Canker growth statistics of the virulent isolate CR of *Endothia parasitica* on seedlings of large surviving (LS) and blight-susceptible (S) American chestnut trees for 1980

Seedling code	Cankers (no.)	Postinoculation canker length (mm) at: 15 days	46 days	Initiation of linear canker growth[x] (days)
S	10	29 a[y]	64 a[y]	21.9 b[y]
LS-20[z]	10	26 ab	59 ab	19.4 b
LS-9[z]	10	21 b	50 bc	21.9 b
LS-23[z]	10	22 b	49 bc	25.2 b
LS-18[z]	10	23 b	48 c	24.6 b
LS-24[z]	10	11 c	26 d	35.3 a

[x]The initiation of linear canker growth was determined as the time after initial lesion (14,15) formation that the increase in canker length from the previous observation exceeded 2 mm. Observations were 2–3 days apart, starting 15 days after inoculation.

[y]Means within columns followed by the same letter are not significantly different ($P < 0.05$) by Duncan's multiple range test. There were five seedlings of each type, with two inoculations per seedling.

[z]Mean and range of diameters (DBH) of parent trees were 46.6 cm and 25–114 cm, respectively. Mean and range of crown rating (percent of crown alive) were 52% and 20–70%, respectively.

TABLE 3. Mean canker lengths and canker severity indexes for 10 large surviving (LS) and seven stump-sprout (SS) American chestnut trees 1 yr after inoculation with virulent *Endothia parasitica* in 1981[x]

Tree code	Cankers (no.)	Mean canker severity index[y]	Mean canker length (mm)
Stump sprouts			
SS 3	6	2,300 a[z]	232 a[z]
SS 5	6	1,386 bc	141 bcd
SS 1	6	1,370 bc	137 cde
SS 7	6	1,348 bc	137 cde
SS 4	6	1,323 bc	132 cde
SS 6	6	1,315 bc	132 cde
SS 2	6	1,107 cd	111 cde
Surviving trees			
LS-19	6	1,780 b	178 b
LS-12	6	1,517 bc	152 bc
LS-1	5	1,408 bc	144 bcd
LS-15	10	774 de	141 cd
LS-9	10	606 e	141 cd
LS-16	10	596 e	103 e
LS-13	10	188 f	123 cde
LS-14	5	182 f	106 de
LS-18	6	122 f	103 de
LS-10	10	66 f	66 f

[x]Results obtained with both *E. parasitica* isolates (CR and WK) were pooled for performing Duncan's multiple range test, as canker lengths produced by both isolates were not significantly different and limbs or portions of limbs on some trees died from natural cankers and/or shading early in the course of the experiment. The latter precluded canker measurement.

[y]Mean of canker length × canker superficiality rating (see text).

[z]Means followed by the same letters are not significantly different, according to Duncan's multiple range test ($P < 0.05$).

Blight-resistance trials on excised stems. In one trial, mean canker lengths after 37 days on excised stems from two surviving trees (LS-13 and LS-1) (Fig. 1) were significantly shorter than mean canker lengths on excised stems from a control stump-sprout tree (Table 4). In a second trial, mean total canker length after 46 days on excised stems from only one surviving tree (LS-10) was significantly less than mean canker lengths on excised stems from two control stump-sprout trees (Table 4). Mean net canker lengths on excised stems from all four surviving trees were significantly different from mean net canker lengths on excised stems from only one control tree (Table 4).

Virulence groups. Low-virulence *E. parasitica* isolates were distinguished on the basis of mean total canker length (≤50 mm for 1980 and 1981, and ≤40 mm for 1979) and superficiality (Fig. 2) of canker development (three or more superficial cankers [>50% of the bark area at the vascular cambium, below the canker surface, with no necrosis] among the five replicates). Intermediate-virulence isolates were established on the basis of mean total canker length (≤100 mm in 1980 and 1981, and ≤70 mm in 1979) with two or more of the five replicate cankers exhibiting superficial canker development. All other isolates were considered virulent. Mean net canker length was used to confirm these determinations, and

TABLE 4. Mean canker lengths on excised stems of large surviving (LS) and stump-sprout (SS) American chestnut trees after inoculation with virulent *Endothia parasitica* isolates CR and WK in 1979 and in 1980

Tree code	Cankers (no.)	Canker length: Total (mm) WK	Total (mm) CR	Net (mm)[x] WK	Net (mm)[x] CR
1979, WK or CR					
SS-8	6	115 a[y]	118 a[y]	94 a[y]	95 a[y]
LS-1[z]	6	88 b	83 b	66 b	65 b
LS-13[z]	8	58 b	62 b	35 c	42 b
1980, WK and CR					
SS-9	5	102 a[y]		83 a[y]	
LS-11[z]	12	83 ab		55 b	
LS-9[z]	12	78 ab		59 b	
SS-10	6	72 bc		51 c	
LS-12[z]	12	52 cd		25 d	
LS-10[z]	12	48 d		33 cd	

[x]Determined by subtracting initial lesion size estimate, after 18 days, from total lesion size at 37 days.

[y]Means within columns followed by the same letters are not significantly different by Duncan's multiple range test ($P < 0.05$). The canker lengths of CR and WK isolates were pooled in 1980 as they were not significantly different by an F-test ($P > 0.05$), and there were some inoculation failures on the SS-9 and SS-10 stems, which were associated with other fungi.

[z]Mean and range of parent tree diameter (DBH) were 65.2 cm and 35 to 114 cm, respectively. Mean and range of crown ratings (percent crown alive) were 50% and 20–95%, respectively.

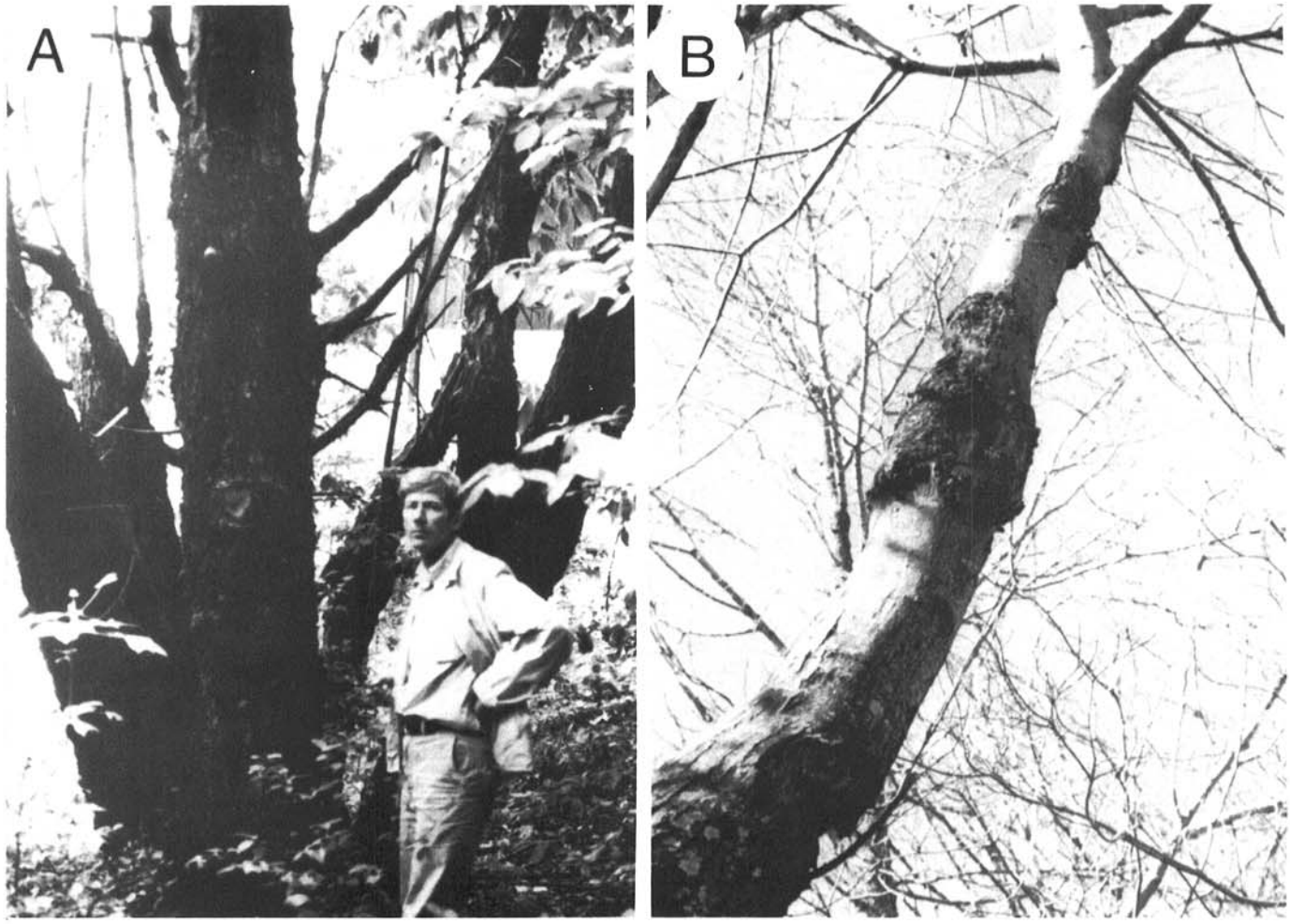

Fig. 1. Large, surviving American chestnut trees: **A,** LS-1 multi-stem tree, having continuous, swollen (note central stem), superficial cankers that encircle the lower stems for about 4 m, in Fairfax County, VA; **B,** limb of LS-13 tree, showing discrete, callused, and swollen cankers with little exposed xylem at the canker centers, in Floyd County, VA.

agreement was found in most cases. Univariate cluster analysis (23) was used to help develop the canker length criteria. This procedure, based on an analysis of variance, groups means having a high degree of affinity to each other, and can be used when the number of treatments is large. Figures 3 and 4 do not represent this statistical procedure, but are frequency distributions that show the structure of the *E. parasitica* populations based on canker length classes. Almost all (97.5%) of the low-virulence isolates, according to canker length, also met the criterion for superficial canker development, and approximately 95% of the virulent isolates produced one or no superficial cankers. Greater disparity between these two criteria was found among the intermediate isolates, however. Swelling of cankers was typically associated with short, superficial cankers.

Cluster analysis results ($P<0.05$) indicated that for mean total canker length, four virulence groups (≈low virulence, ≈intermediate virulence, ≈virulent-A, and virulent-B) were recognized in 1979 (surviving trees), 1980 (surviving trees), and 1981 (stump sprouts). Cluster breaks occurred above 30–40, 60–70, and 100–110 mm in 1979, above 40–50, 90–100, and 130–140 mm in 1980, and above 40–50, 100–110, and 130–140 mm in 1981. For the 1980 and 1981 populations of *E. parasitica*, the low-virulence cluster occurred at the same canker length range (≤50 mm), but in 1979, the low-virulence canker length range (≤40 mm) occurred at one frequency unit lower. Similarly, the CR virulent control isolate produced a shorter mean total canker length in 1979 than in 1980 and 1981. In all years, the WK and CR virulent control isolates were located at the peak of the frequency distribution curves (Fig. 3). Four virulence groups ($P<0.05$) were found also for mean net

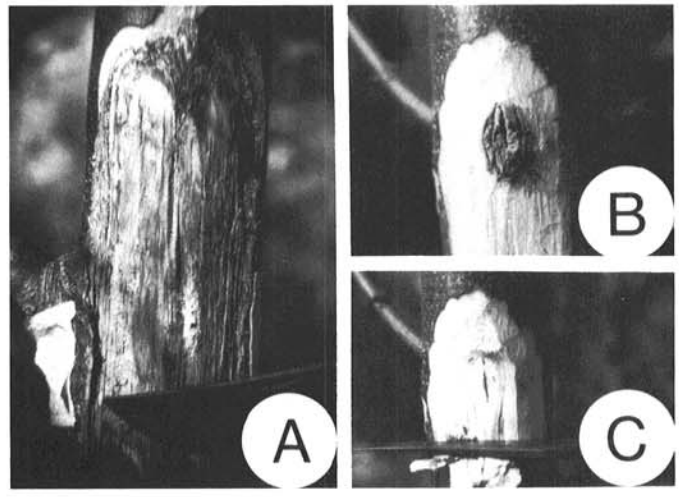

Fig. 2. Cankers produced by a virulent isolate (from LS-18 tree) and a low-virulence isolate (from LS-10 tree) of *Endothia parasitica* after approximately 4 mo on the same American chestnut stump sprout: **A,** dissected large, nonsuperficial canker, produced by the virulent isolate, showing necrosis and discoloration in the xylem; **B,** small, superficial canker, produced by the low-virulence isolate, with periderm cut away, exposing the secondary phloem, to show clearly the canker size; **C,** same canker as in B, but dissected to the xylem to show absence of necrosis and discoloration beneath canker surface. Length of canker shown in B was 19 mm, and length of canker shown in A was 125 mm. A piece of masking tape is present (at the tip of the knife) in the left side of photograph A.

canker lengths in 1979, 1980, and 1981. Cluster breaks occurred above 15–20, 40–45, and 60–65 mm in 1979, above 10–15, 30–35, and 50–55 mm in 1980, and above 10–15, 50–55, and 60–65 mm in 1981. Trends were similar to those found for total canker length, except that the virulence group length criteria for 1979 were shifted to the right (greater length), relative to 1980 and 1981 (probably due to a 15–17 day longer net growth period in 1979 than in 1980 and 1981), the CR isolate was shifted to the right of the frequency peaks, and the second and third virulence groups for 1980 were shifted somewhat to the left (shorter length), relative to 1981. These analyses showed also that some cankers did not grow or grew very little during the latter part of the growing season (Fig. 4).

Reduced colony pigmentation on PDA usually was not associated with low-virulence and intermediate isolates in all years, and some virulent isolates had greatly reduced pigmentation; >50% of the colony surface was white after 7–10 days. For example, in 1979, three of five low-virulence isolates tested had normal, regularly zonate pigmentation typical of *E. parasitica*, through several transfers, when examined after 10 days of incubation at 27–28 C in room light (cool-white fluorescent). Two isolates were white. Six of 16 virulent isolates and none of four intermediate isolates were white in the same trials. Ten of the 133 isolates assayed in 1979 virulence trials were selected for reduced pigmentation and other cultural abnormality, but no relationship to pathogenicity was found.

Assays for dsRNA (two to three trials per culture) of 12 subcultures from 11 low- or intermediate-virulence *E. parasitica* isolates, obtained from stump sprouts and surviving trees, were done by polyacrylamide-gel electrophoresis using procedures modified from Morris and Dodds (22), in the laboratory of W. L. MacDonald, M. Double, and R. L. Willey (West Virginia University). Low-virulence isolate L-85 (somewhat slow growing) contained one major band and several minor bands of dsRNA; L-90 (appressed colonies, slow growing, and irregular colony margins), L-206, L-186, L-83, L-W2B, L-M8 (irregular colony margins) and L-M6 (irregular colony margins) contained one major band, while intermediate isolate I-183 contained one major band of dsRNA; no bands were found for I-191, I-69, or L-W2A. Similar results were obtained for L-W2A by S. Anagnostakis (Connecticut Agricultural Experiment Station). L-W2A and L-W2B were daughter subcultures (mass transfer) of low-virulence isolate L-W2. Except as noted, these isolates grew at a normal rate (12), and at 7–10 days had regular margins, were not appressed, and were pigmented light to dark yellow-orange in regularly zonate colonies.

Frequencies of low-virulence and intermediate-virulence *E. parasitica* isolates found on large, surviving American chestnut trees. Of 103 *E. parasitica* isolates obtained from the northern Virginia LS-1 tree (Fig. 1A) in 1979, 19.4% had low virulence, and 12.6% had intermediate virulence (Table 5). Low-virulence isolates were found over all portions of the tree (Table 6), but the greatest percentage was associated with the continuous, lower stem cankers that completely encircled the entire stems of this multi-stem tree (Fig. 1A); by a G-test (25) the frequency of isolate type was not independent ($P<0.05$) of stem height. These swollen cankers were almost completely superficial, as there was little or no exposed xylem, and all cork-borer, bark samples showed a healthy layer (about 0.5–0.8 cm thick) of inner bark between the inner radial margin of the cankers and the vascular cambium. Cankers of this type were commonly observed on surviving trees. The three most pathogenic isolates obtained from this tree also were located in the lowest stem zone, and a low-virulence isolate was found on a discrete canker as high as 12.8 m above the ground. In a 1980 test

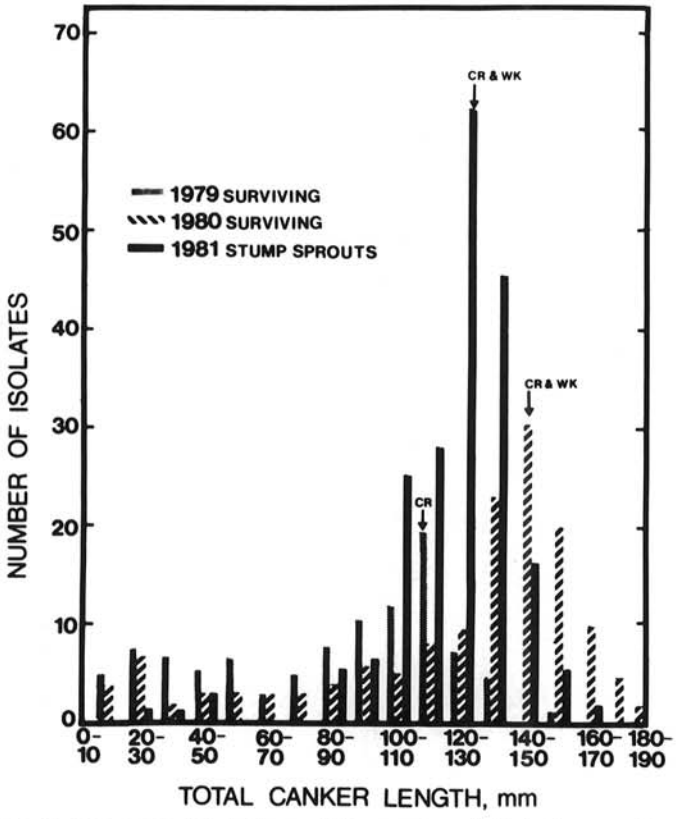

Fig. 3. Frequency distributions of the number of *Endothia parasitica* isolates from large, surviving (1979 and 1980) and small, stump-sprout (1981) American chestnut trees that produced total canker lengths on stump sprouts in the classes 0–10 mm to 180–190 mm. Positions of virulent control isolates, CR and WK, are shown. Variance components for tree, isolate and error were 283, 1,126, and 548 in 1979; 159, 1,613, and 689 in 1980; and 360, 135, and 476 in 1981.

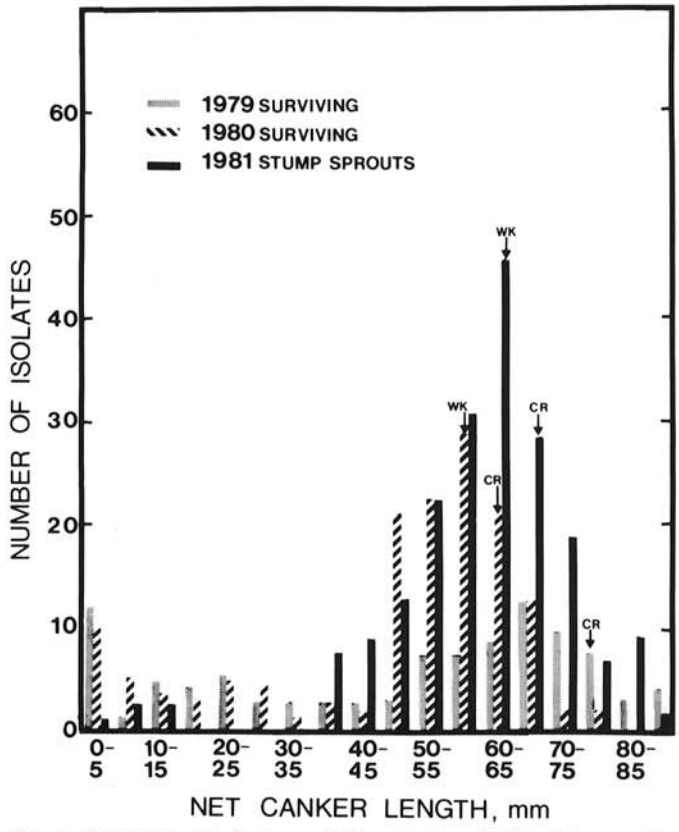

Fig. 4. Frequency distributions of the number of *Endothia parasitica* isolates from large, surviving (1979 and 1980) and small, stump-sprout (1981) American chestnut trees that produced mean net canker lengths (October measurement minus August measurement) on stump sprouts in the classes 0–5 mm to 85–90 mm. Positions of virulent control isolates, CR and WK, are shown. Variance components for tree, isolate, and error were 171, 567, and 474 in 1979; 159, 1,613, and 689 in 1980; and 360, 135, and 476 in 1981.

TABLE 5. Percentage of *Endothia parasitica* isolates associated with large surviving (LS), stump-sprout (SS), and associated trees (AT) of American chestnut that had low and intermediate virulence[a]

Trees	State (counties)	DBH (cm)/ crown rating (%)	Isolates tested per trees sampled (no./no.)	Isolates with low virulence (%)	Isolates with intermediate virulence (%)
Stump sprouts[b]					
A	VA (Floyd, Giles, Craig, Montgomery); WV (Summers, Pendleton)		114/114	4	3
B	PA (Lycoming, Snyder, Northumberland)		84/84	0	0
Surviving (LS) and associated trees (AT)					
LS-1	NVA[c] (Fairfax)	39/95	103/1	19	13
AT-1[d]	NVA[c] (Fairfax)	5/100	6/1	0	17
LS-2	NVA[c] (Fairfax)	69/65	12/1	8	8
AT-2[d]	NVA[c] (Fairfax)	8/50	5/1	40	20
LS-3	NVA[c] (Fauquier)	34/95	10/1	10	0
AT-3[d]	NVA[c] (Fauquier)	11/65	6/1	33	33
LS-4	NVA[c] (Fauquier)	38/50	11/1	18	18
LS-5	NVA[c] (Fauquier)	58/40	11/1	0	0
LS-6	NVA[c] (Fauquier)	22/70	10/1	10	10
AT-4[d]	NVA[c] (Fauquier)	14/50	5/1	0	20
AT-5[d]	NVA[c] (Fauquier)	11/50	5/1	0	0
LS-7	NVA[c] (Fauquier)	38/50	10/1	10	0
LS-8	NVA[c] (Fauquier)	32/70	10/1	10	10
LS-9	WV (Summers)	114/20	30/1	0	0
LS-10	WV (Doddridge)	72/60	17/1	18	41
LS-11	WV (Doddridge)	68/60	10/1	20	40
LS-12	WV (Braxton)	63/30	10/1	20	10
LS-13	VA (Floyd)	35/85	10/1	20	0
LS-14	VA (Floyd)	25/95	11/1	0	27
LS-15	VA (Amherst)	102/70	10/1	0	20
LS-16	PA (Dauphin)	68/80	11/1	9	36
LS-17	OH (Coshocton)	81/70	10/1	30	20
LS-18	OH (Coshocton)	39/50	11/1	45	27
LS-19	VA (Albemarle)	38/95	10/1	40	20

[a] Virulence trials were conducted in 1979, 1980, 1981, and 1982 between June and October. Low-virulence and intermediate isolates produced short, superficial cankers compared to virulent isolates (see text).
[b] Mean and range of stump-sprout diameter (DBH) were 5.7 cm and 4.0–17.8 cm, respectively, for VA-WV trees; and 8.6 cm and 4.0–19.3 cm, respectively, for PA trees.
[c] NVA indicates northern Virginia.
[d] Small American chestnut trees located near large, surviving American chestnut trees.

for suppressed low virulence in 12 yellow and five white virulent isolates from the LS-1 tree (using biopsy samples from the 1979 trial), three of the 17 (two yellow and one white) had low virulence, and none had intermediate virulence. Thus, the percentage of low- and intermediate-virulence isolates in the LS-1 tree, adding these values, would be greater than the 32.0% indicated in Table 5.

No low- or intermediate-virulence isolates were found in 1979 among the 30 *E. parasitica* isolates recovered from the West Virginia LS-9 surviving tree (Table 5).

Low- or intermediate-virulence isolates were found on 10 of the 12 northern Virginia American chestnut trees sampled in 1980 (Table 5). Based on a smaller number of *E. parasitica* isolates per tree than used above for the LS-1 tree, the percentage of low-virulence isolates ranged from 0 to 18%, and the percentage of intermediate isolates ranged from 0 to 10% for large, surviving trees. For small trees, located near surviving trees, 0–40% of the isolates had low virulence, and 0 to 33% were intermediate (Table 5). Overall, 19.9% of the 101 *E. parasitica* isolates from the 12 trees had low or intermediate virulence.

Many of the other large, surviving American chestnut trees, examined in the blight resistance trials, were infected with low- (Fig. 2) or intermediate-virulence strains of *E. parasitica*, and combined percentages were sometimes greater than 50% (Table 5).

Frequencies of low-virulence and intermediate-virulence *E. parasitica* isolates found on small, stump-sprout American chestnut trees. Overall, 2.5% of 198 *E. parasitica* isolates recovered from American chestnut stump sprouts in Virginia, West Virginia, and Pennsylvania in 1981 had low virulence, and 1.5% were intermediate. All of these were found in Virginia-West Virginia and

TABLE 6. Percentage of *Endothia parasitica* isolates, associated with different stem heights on one large, surviving American chestnut tree (LS-1), that had low and intermediate virulence in 1979[a,b]

Stem height above ground (m)	Isolates tested (no.)	Low-virulence isolates (%)	Intermediate-virulence isolates (%)
0– 3.0	41	29.3	9.8
3.0– 6.0	10	10.0	0.0
6.0– 9.0	24	8.3	20.8
9.0–13.0	28	7.1	21.4

[a] This tree (Fig. 1A) is a multi-stem tree (largest stem DBH = 39.4 cm) and had a crown rating (percent crown that was alive) of 95% in 1979.
[b] The frequency of isolate type was not independent ($P<0.05$) of stem height by an RC test of independence using the G-statistic.

comprised 7.0% (4.4% low virulence and 2.6% intermediate) of the Virginia-West Virginia isolates (Table 5). Two of five low-virulence and one of three intermediate isolates were obtained from trees growing in recently (<15 yr old) clear-cut forest areas. However, only 27 of 198 trees sampled were growing in these areas. The remainder were obtained from mature or nearly mature (pole-timber) forest areas. Thus, 11.1% of all isolates recovered from clearcut areas had low or intermediate virulence. When several sprouts in a clump were sampled, no more than one low-virulence or intermediate isolate was obtained per clump.

Stromata production by low-virulence, intermediate-virulence, and virulent isolates of *E. parasitica*. Stromata production on

stump sprouts by low-virulence isolates in all years was significantly ($P<0.05$) less than for intermediate or virulent isolates (Table 7). For virulent isolates, stromata production was greatest in 1980 and least in 1982. In 1980, a severe drought occurred, and 1982 was characterized by above-average rainfall during the growing season.

DISCUSSION

These results provide evidence that blight resistance exists in the population of large, surviving American chestnut trees. This resistance may contribute, in part, to the survival of these trees. Also, one tree (LS-9) may survive only because of blight resistance (mainly superficial canker development), as no evidence was obtained to indicate that low virulence in *E. parasitica* contributes to the survival of this tree. Additional research on this tree, now in poor condition (Table 5), is required to support this hypothesis, however. Evidence also suggests that blight resistance in American chestnut may be heritable. For example, mean canker lengths on LS-18 bark-grafted scions, mean canker lengths on LS-18 seedlings, and the mean canker severity index on the parent LS-18 tree were all significantly different from the American chestnut controls. The superficial development of many cankers on large, surviving trees in the in situ trials suggests that this is an important factor, in addition to canker length, in identifying blight resistance in surviving trees. Location differences did not appear to be critical factors in the in situ trial results, since a greater range in mean canker length was obtained at the stump-sprout location than among all other locations.

In extensive blight-resistance trials with European chestnut trees, Bazzigher and Schmid (3,5) found that stem diameter and growth of the pathogen in host tissue were the two critical factors determining tree mortality. Length of canker was a reliable measure of growth in host tissue and blight resistance. In addition, Bazzigher (4) reported that European chestnut trees were highly susceptible to blight up to the ages of 6–7 yr; thereafter, resistance increased until about 20 yr, and subsequently declined slowly. Thus, small-stem, juvenile tissues are most susceptible to mortality. A similar situation may exist in the American chestnut, and, together with the thin bark, may be a factor in the early kill of young seedlings and grafts in the blight-resistance trials. In contrast, the larger and thicker-bark branches on the older, surviving trees used in the in situ blight-resistance trials typically were not dead after 1 yr, for trees having low mean canker severity indexes.

Low canker severity indexes obtained in the in situ trials and survival of American chestnut trees may be due to resistance induced by natural cankers existing on the trees before inoculation. Arguing against this, however, is the rapid killing of American chestnut stump sprouts in forest clearcuts (14) and during the original blight pandemic. These trees typically had multiple cankers of various sizes and ages, and the first canker formed did not cause the trees to survive. Also, three surviving trees in the in situ blight-resistance trials were as naturally cankered as other surviving trees, yet high canker severity indexes and branch death were observed for these trees.

Histopathological evidence supports the results of the in situ blight-resistance trial. Hebard (14) found that host reactions and mycelial fan development by the virulent CR isolate in the LS-13 tree (direct inoculation) were similar to those in blight-resistant Chinese chestnut (cv. Nanking) and dissimilar from those in American chestnut stump sprouts. In both the LS-13 tree and Chinese chestnut tree, only one superficial fan typically developed, whereas multiple fans, one often at the vascular cambium, developed in the stump sprouts.

How much blight resistance is due to genetic, ontogenetic, and environmental factors requires further study. Thus, it is not clear how useful blight resistance will be in a breeding program to develop blight-resistant American chestnut trees, which is the goal of several research programs (6,9,27). The LS-18 tree may be useful in achieving this goal, if the postulated blight-resistance gene(s) in this tree can be combined with blight-resistance genes from other large, surviving American chestnut trees. No single large, surviving American chestnut tree examined by us has sufficient blight resistance to be useful in outplantings. Additionally, many large, surviving American chestnut trees are found on desirable sites and are relatively free from competition from other trees. Thus, the role of environment in blight resistance of these trees should be evaluated. High soil mineral nitrogen (>5 μg NO_3^- per gram of soil) and lack of shading from neighboring trees have been associated with some, but not all, surviving trees (G. J. Griffin, *unpublished*), and environmental factors, such as low temperature stress and branch shading, have been associated with lowered blight resistance in Oriental chestnut species (18,28). Many branches inoculated in the in situ blight-resistance trials were low on the trees, where shading and self-pruning are typically greatest. This may have affected host resistance and canker lengths in some trees, such as LS-1 and LS-13, which had extensive shading from neighboring trees or higher branches. Some blighted large trees, such as the LS-18 tree and the LS-10 tree, survive, although they are subjected to stress on roots due to cattle (soil compaction) and steep slope (soil erosion), respectively. In the presence of these stresses, these two trees still had the lowest canker severity indexes of all surviving trees examined.

Most isolates of *E. parasitica* obtained from large, surviving American chestnut trees were virulent, as found previously (12). However, unlike our previous finding, most surviving trees were infected with one or more low-virulence or intermediate strains. This difference was probably due to the larger number of isolates of *E. parasitica* obtained per surviving tree in the present study. In agreement with Jaynes and Elliston (17), we equate low virulence with hypovirulence, whether the determinants are cytoplasmic or nuclear, but separate intermediate-virulence isolates for the present. Future research may alter the latter tentative label. In their 1957 glossary, Snell and Dick (26) defined virulence as "degree of pathogenicity" and hypo- as "lower" or "under," and this is the basis for our use of terms. In most cases of our study, the incidence of hypovirulent and intermediate strains per surviving tree was less than 50%, but was as high as 73%. For one large, surviving tree in Tennessee, Kuhlman (19) recently reported that 36% of 78 *E. parasitica* isolates were hypovirulent. Isolates were labeled hypovirulent based on colony growth characteristics (colony color, growth rate, and amount of aerial mycelium [E. G. Kuhlman, *personal communication*]) and in vitro conversion trials for a transmissible factor. In the present study, all hypovirulent isolates contained dsRNA in one or more of the two to three trials. That two intermediate *E. parasitica* isolates were not positive for dsRNA suggests that either the titer of dsRNA was below detectable levels or that nuclear determinants may be involved. Jaynes and Elliston (17) present similar findings for some hypovirulent strains. Subculture L-W2B of hypovirulent isolate L-W2 was positive for dsRNA but subculture L-W2A was not, and this suggests that negative results for dsRNA do not rule out that dsRNA is not associated with a given canker isolate of *E. parasitica*.

TABLE 7. Stromata production indexes for low-virulence, intermediate-virulence, and virulent isolates of *Endothia parasitica* on American chestnut stump sprouts from 1979 to 1982

Isolate type	Mean stromata production index[x]			
	1979	1980	1981	1982
Low-virulence	0.13 c[y] (20)[z]	0.96 c[y] (15)[z]	0.28 c[y] (5)[z]	0.28 c[y] (15)[z]
Intermediate-virulence	0.73 b (13)	2.70 b (16)	1.07 b (3)	1.05 b (22)
Virulent	3.14 a (100)	3.79 a (107)	2.60 a (190)	1.67 a (27)

[x] Production of stromata on each canker was rated visually approximately 4 mo after inoculation according to the following scale: 0 = none, 1 = trace, 2 = slight, 3 = moderate and 4 = abundant. Five replicate cankers were usually rated per isolate.

[y] Mean stromata indexes followed by the same letter, within columns, are not significantly different, according to Duncan's multiple range test ($P<0.05$).

[z] Number in parentheses indicates the number of *E. parasitica* isolates rated.

For some trees, such as LS-12, LS-1, and LS-19, hypovirulent or intermediate strains may be important, or even critical, to survival, as there was little or no evidence of blight resistance in these trees. The LS-1 tree, for example, showed blight resistance in only one test, and approximately one third of the 103 isolates from this tree were hypovirulent or intermediate. However, some of the virulent isolates from this tree showed suppressed hypovirulence (or hypovirulence instability), as found previously for one isolate from this tree (12), which would raise the percentage of potential hypovirulent and intermediate isolates recovered from this tree. The one-third value, close to that found overall for surviving trees, is more than four times the incidence (7.0%) of hypovirulent and intermediate strains found in the general population of *E. parasitica* recovered from Virginia-West Virginia stump sprouts; this was a highly significant difference ($P<0.005$) by a G-test (25). The incidence also was significantly higher ($P<0.005$) on the remainder of the large, surviving trees than on the stump sprouts. This indirect evidence must be qualified, however, by noting that larger, presumably older trees probably had a greater length of time to become infected with hypovirulent strains. Also, the older (lower) portions of the LS-1 tree had a significantly greater incidence of hypovirulent (but not hypovirulent plus intermediate) strains than younger portions.

Blight resistance may allow time for trees, such as LS-13, LS-18, and LS-10, to become infected with hypovirulent strains. Local dissemination of hypovirulence determinants (cytoplasmic or nuclear) in nature appears to be slow (2,10,29) and this may be a problem for achieving biological control of chestnut blight in North America. Shain (24) suggests, however, that slow local spread need not preclude success of hypovirulent strains. Hypovirulent and intermediate strains produced much lower amounts of stromata than virulent strains, and hypovirulent strains often lack production of ascospores (17); this may greatly restrict dissemination, relative to virulent strains. Vegetative incompatibility (1,20,21) may slow further the spread of cytoplasmic, and potentially nuclear, hypovirulence determinants on trees or within cankers, once a tree is infected with hypovirulent strains. Vegetative incompatibility between hypovirulent and virulent strains appears to be an important factor in the LS-1 tree (G. J. Griffin, *unpublished*). Thus, some degree of blight resistance in surviving trees in the natural range may allow these trees to withstand the virulent inoculum produced on stump-sprout American chestnut trees (14), until hypovirulent strains are disseminated to and established in these trees. An exception to this would be instances in which small trees grow near other trees, especially large trees, already infected with hypovirulent strains. Thus, we found that small trees in northern Virginia often had as great or greater an incidence of hypovirulent and intermediate strains as did large trees that were located nearby.

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