Developmental Histopathology of Cankers Incited by Hypovirulent and Virulent Isolates of *Endothia parasitica* on Susceptible and Resistant Chestnut Trees

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ABSTRACT

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The histopathology of canker development on blight-resistant Chinese and blight-resistant and blight-susceptible American chestnut was examined after wounding plus inoculation with virulent (V) or hypovirulent (H) isolates of *E. parasitica*. Bark lesions or wounds were delimited initially by a zone of lignified tissue forming 8–10 days after inoculation in all host-treatment combinations, which halted advance of individual hyphae. Wound periderm formation began immediately adjacent to this lignified zone 10–14 days after inoculation. Wound periderm formation began at the deepest point of a wound or canker and progressed outward to the bark surface. The development of wound periderm was affected by its orientation in bark tissues and was disrupted by growth of mycelial fans. Otherwise, there were no differences in rate or extent of wound periderm formation among host-treatment combinations. Superficial cankers were formed in resistant trees by V *E. parasitica*, or in susceptible trees by H *E. parasitica*. This occurred when slow-growing mycelial fans expanded through outer regions of bark, where wound periderm had not formed or had not developed fully. The lignified zone and developing wound periderm in the susceptible host were penetrated by mycelial fans of the V isolate at 18 days and by mycelial fans of the H isolate at 28 days. In the two resistant hosts, mycelial fans of V but not H *E. parasitica* sometimes formed and grew after 30 days. Physical pressure probably played a role in the growth of mycelial fans. Mycelial fan penetration halted wound periderm development because host tissues were killed; individual hyphae or fans killed host cells before touching them.

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

One of the difficulties in breeding timber-type chestnut trees for resistance to blight is the lack of rapid means of screening young (about 1-yr-old) seedlings for blight resistance (19). Several workers have identified biochemical markers associated with blight resistance (12,18,25,31), but a direct relation to resistance has not been established. Hebard and Kaufman (18) attempted to identify resistance in chestnut callus tissue cultures by challenging them with *Endothia parasitica* (Murr.) P. J. & H. W. And., but detected no differences in rates of colonization of resistant and susceptible tissues. Knowledge of the histopathological characters which distinguish resistance reactions from susceptibility reactions in intact plants would assist interpretation of the reactions in tissue cultures and young seedlings and would help elucidate the biochemical basis of resistance. This information also would help determine why some large American chestnut trees have survived long-term infections by *E. parasitica* and why superficial cankers (those not reaching the vascular cambium) are formed by hypovirulent isolates of the fungus.

The histopathology of blight cankers has been examined on blight-susceptible American chestnut, *Castanea dentata* (Marsh.) Borkh. (7,21), on slightly blight-resistant European chestnut, *C. sativa* Mill. (3,6,14,15), and on moderately to highly blight-resistant Japanese chestnut, *C. crenata* Sieb. & Zucc. (35). We know of no reports of the histopathology of blight cankers on highly blight-resistant Chinese chestnut, *C. mollissima* Blume, which is commonly planted in the eastern United States. All of the above work suggested that canker enlargement occurred only when mycelial fans were present, except for a specialized case on Japanese chestnut (35). Also, wound periderm appeared to be central to resistance or hypovirulence reactions. However, it was not clear whether the rate or extent of wound periderm formation or the rate of fungal development was crucial to canker enlargement. To investigate this we have examined the histopathology of canker development in blight-resistant and blight-susceptible chestnut trees after inoculation with a virulent strain and a hypovirulent strain of *E. parasitica*.

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MATERIALS AND METHODS

Inoculations. Three types of chestnut were inoculated with *E. parasitica*: (i) Stump sprouts of *C. dentata* growing in the understory at a site forested with small sawtimber. The sprouts were located in the Jefferson National Forest near Blacksburg, Virginia. They were 2–3 cm in diameter at breast height (1.4 m) (dbh) and were approximately 10 to 20 yr old. They are typically susceptible to blight (16) (S trees). The sprouts were inoculated from ground level to a height of 2 m. (ii) Scions of *C. mollissima* 'Nanking' grafted to seedling rootstock of *C. mollissima*. These were located at the VPI & SU Horticultural Farm in Blacksburg, Virginia. The scions were approximately 25 yr old. Cultivar Nanking has demonstrated field resistance to blight (27) (R trees). Cultivar Nanking is *not* PI 58602 from Nanking, China (27). On each tree, one horizontally oriented branch, 3–6 cm in diameter, was inoculated. These were partially shaded by the upper canopy and neighboring trees. (iii) A large (35 cm dbh), surviving American chestnut tree, located in Floyd County, Virginia. This tree has been shown to be slightly to moderately blight resistant (16,17) (MR tree). Blight-free branches 3–6 cm in diameter were inoculated. These were partially shaded by the upper canopy and neighboring trees.

Each type of tree was wounded to the phloem, but not the vascular cambium (~1 mm deep) (Fig. 1A) with a cork borer (0.6 cm diameter), and a disk of bark was removed. Each wound was treated by not inoculating it (NI) or inoculating it with the virulent (V) isolate, CR, or with the dsRNA-positive, hypovirulent (H)

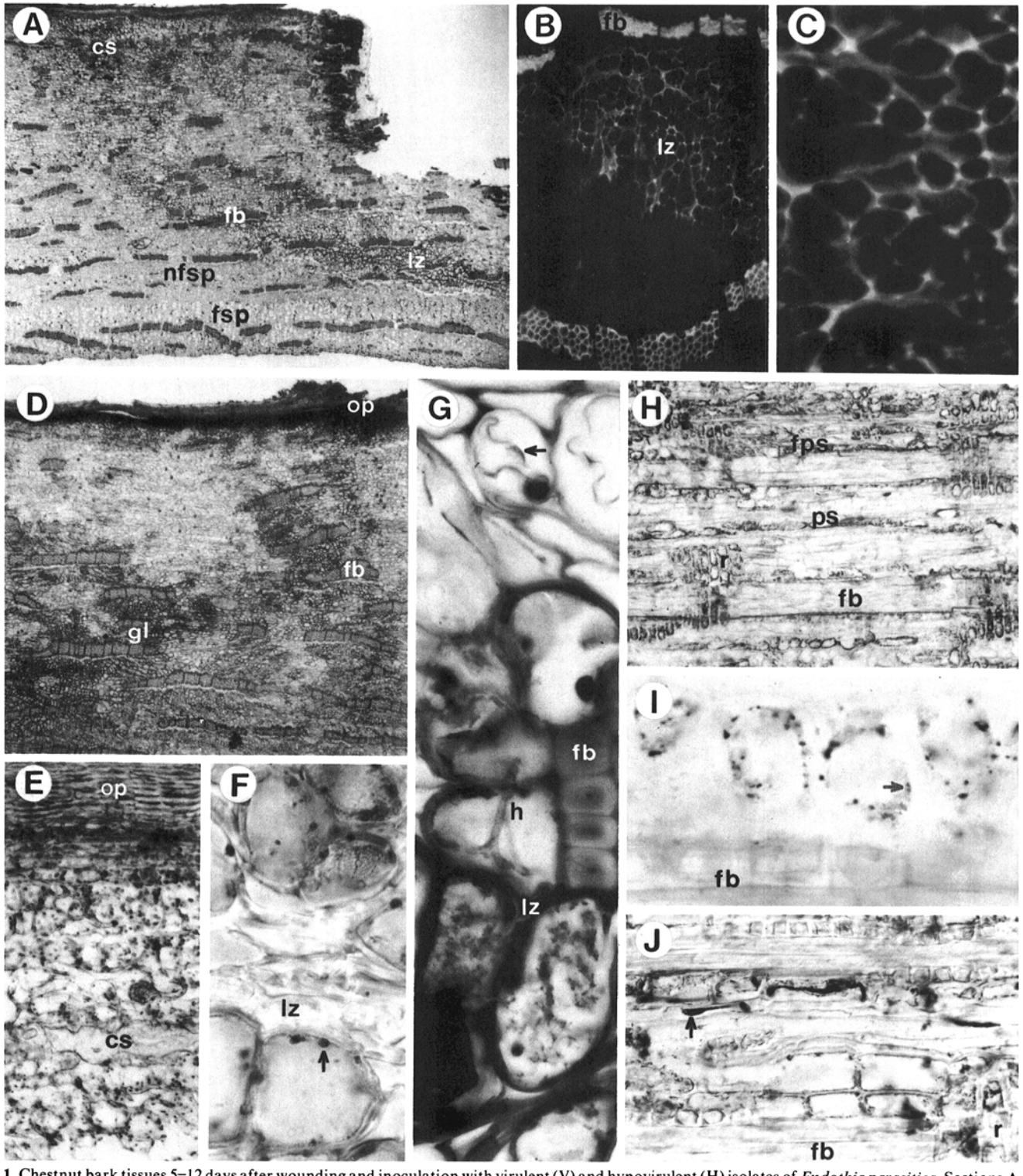

Fig. 1. Chestnut bark tissues 5–12 days after wounding and inoculation with virulent (V) and hypovirulent (H) isolates of *Endothia parasitica*. Sections **A–C** and **G** were stained with safranin and fast green. **A,** Day 12. Transverse section of moderately blight-resistant American chestnut wounded and inoculated with V isolate. Inoculation site is on right top of section. Functioning (fsp) and nonfunctioning (nfsp) secondary phloem are evident. Infected tissues are surrounded by a lignified zone (lz), staining darkly with safranin, which extends from the point marked lz, through the fiber bundle (fb) and cortical sclereids (cs) to the outer periderm (×17). **B,** Section of Fig. 1A. Fluorescence under UV light of lignified zone (lz) and fiber bundles (fb) indicates lignification of cell walls (×100). **C,** Detail of Fig. 1B. Prominent fluorescence at cell corners indicate lignification (×340). **D,** Day 10. Transverse section of blight-resistant Chinese chestnut cv. Nanking with V isolate. Intense stain for β-glucosidase activity (gl) occurs in the zone of lignification reaching from the point marked gl to the fiber bundle (fb) and up to the outer periderm (op). Stain for activity also occurs in the lumen of chlorenchyma underneath the outer periderm, on both sides of the zone of lignification (×29). **E,** Day 10. Radial section of Chinese chestnut inoculated with V isolate. Stain for phenolase activity in lignifying zone is in plastids of cortical parenchyma under outer periderm (op). Note cortical sclereids (cs) (×165). **F,** Partially crossed polarizers. Canker of Fig. 4E. Stain for phenolase in plastids (arrow) of parenchymatous elements in lignifying zone (lz) of secondary phloem. Thick-walled cells fluoresced under UV light, indicating lignin (×710). **G,** Detail of Fig. 1A at border of infected tissue and lignified zone (lz). Note hypha (h) extending up to, but not through, lignified zone, and plasmolysis, at arrow, indicating cell death in infected tissue (×770). **H–J,** Radial sections of American chestnut stained for diaphorase activity. **H,** Day 5. Moderately blight-resistant American chestnut with V isolate. Uninfected tissue. Stain for diaphorase activity is present in axial parenchyma strands (ps and fps) between fiber bundles (fb) and in rays (r), indicating living cells (×135). **I,** Day 5. Blight-susceptible American chestnut stump sprout with V isolate. Uninfected tissue. Stain for diaphorase activity, probably in mitochondria (arrow), of ray-associated parenchyma next to fiber bundle (fb) (×1,070). **J,** Interference contrast. Same canker as Fig. 1H. At advancing edge of infection. Stain for diaphorase activity is present in hyphae (such as at arrow) but absent in ray cells (r) and cells of axial parenchyma strands (such as next to fiber bundle, fb), indicating host cell death (×370).

isolate, Ep66 (16). This gave nine host-treatment combinations: S-V, S-H, S-NI, MR-V, MR-H, MR-NI, R-V, R-H, and R-NI. Wounds were spaced approximately 15 cm apart. Mycelial ball inoculum (4) (~0.5 cm diameter) was grown at room temperature (approximately 27 C) in 75 ml of liquid medium (30) in 250-ml Erlenmeyer flasks on a gyrorotary shaker (New Brunswick Scientific Co., Inc., New Brunswick, NJ 08817) at 120 rpm. Balls were washed with sterile water prior to use. After treatment, each wound was covered with masking tape, which was left on until sampling.

Two major time-course experiments were conducted, one in 1978 and one in 1979. A preliminary experiment was conducted in 1977 to develop sampling guidelines. Inoculations for the 1978 experiment were made on 3 July. From 10 to 30 days after inoculation, two samples were collected every 2–3 days. One of the samples was sectioned for microscopy and one processed for Mullick's (28) F-F test. During the second and third months, single samples, for microscopy only, were collected weekly from the S and R trees and approximately biweekly from the MR tree. Not all the required inoculations could be fitted on a single S tree or the single inoculated branch of an R tree. Samples were collected from one tree until there were no more on that tree, and then sampling was begun on a new tree. During the first 30 days in 1978, on a day when the potential samples on an S or R tree would be exhausted, duplicate samples were collected, one from the old tree and one from a new tree. This occurred every other sample on S trees and once at 30 days for R trees. Eight S and four R trees were inoculated in 1978. Inoculations for the 1979 experiment were made on June 7. From 2 to 20 days after inoculation, single samples were collected every 3 days. Two additional samples were collected, one at 46 and one at 81 days after the inoculation in 1979. These samples were used for microscopy; additional single samples for Mullick's (28) F-F test were collected from the R-V combination between 2 and 20 days after inoculation. In 1979, all required samples for the first 20 days of the experiment could be fitted on one S or R tree, so no duplicates were collected. Two S and two R trees were inoculated in 1979.

The experimental design was a split, split-plot, but we could establish only one block because of the separation of the trees, the uniqueness of the MR tree and the time required to grow plots of trees (approximately 5–10 yr). However, since artificially induced cankers on American chestnut vary in size as much within as among trees (16), and since the Chinese chestnuts were a grafted cultivar, population variances could be estimated from the individual cankers. We also accounted for variation among the trees by collecting from two trees on days when new trees were sampled, as indicated. Thus, the experiments were analyzed as a completely randomized design. This is conservative for within-tree comparisons of isolates. Analysis of covariance was used when regressions over time were significant. Means were separated by Duncan's multiple range test, where appropriate.

Sampling, tissue preparation, and staining. Samples consisted of 2 × 3 to 3 × 5 cm rectangular patches of bark containing an entire canker, except when cankers on the S trees grew larger than approximately 5 cm long. In that case, 3 × 5 cm rectangular bark

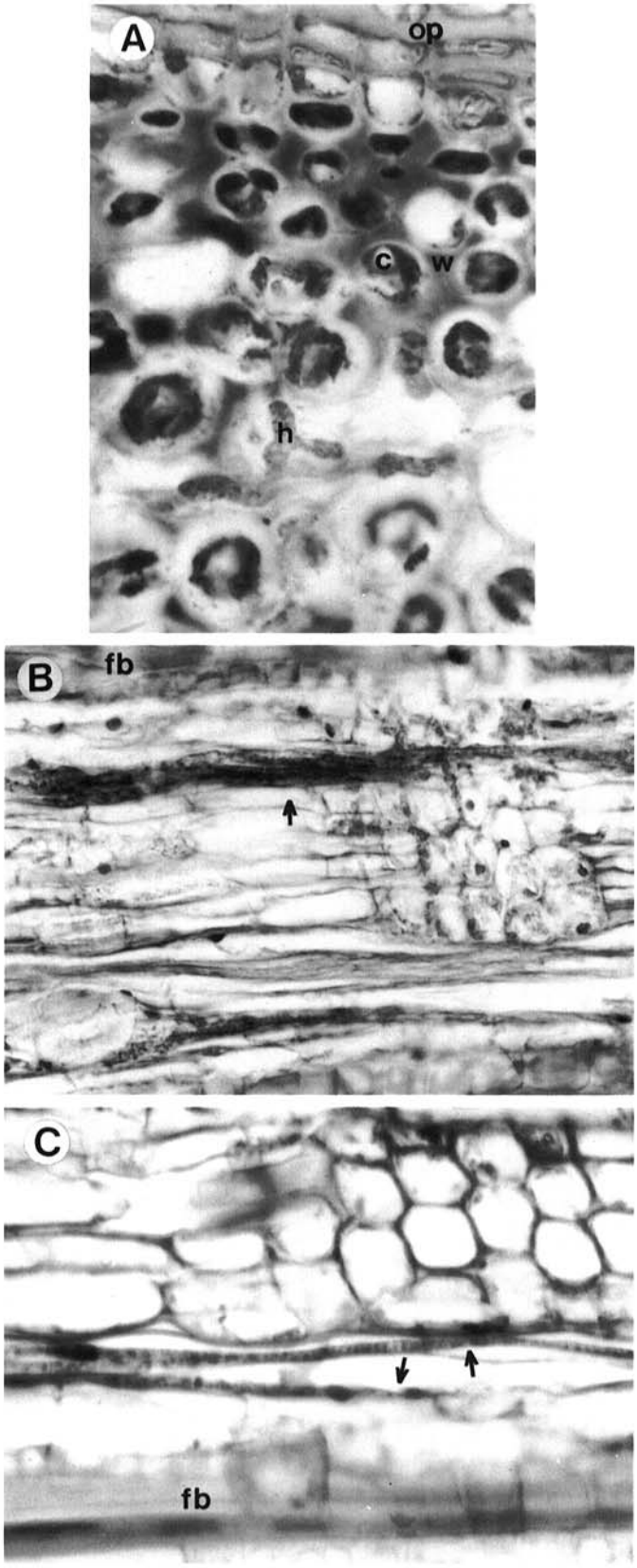

Fig. 2. Radial sections of Chinese and American chestnut trees inoculated with a virulent isolate of *Endothia parasitica* showing host esterase activity in the presence of hyphae (A) and hyphal aggregates (B-C), possible forerunners of mycelial fans. Selected fiber bundles (fb) noted to give perspective. **A,** Day 2. Radial section of blight-resistant Chinese chestnut cultivar Nanking. Stain for esterase activity is found in walls (w) as well as cytoplasm (c). This is especially evident in thick-walled chlorenchyma under outer periderm (op). In Chinese, but not American chestnut, stain for wall-localized activity persisted when hyphae (h) approached the cells (× 770). **B–C,** Sections stained with safranin and fast green. **B,** Day 25. Blight-resistant Chinese chestnut showing a mature hyphal aggregate (arrow). Intercellular hyphae surround some of the ray cells to the right of the arrow (×330). **C,** Day 14. Blight-susceptible American chestnut stump sprout, showing a young aggregate of two long, intercellular hyphae (arrows) (×470).

patches included the uncolonized margin of the canker. The patches included all portions of the bark from the vascular cambium outward.

For microscopy, the samples were subdivided into four rectangular pieces, each piece containing a portion of the inoculation hole. Under vacuum, one piece was fixed in 10% acrolein, dehydrated (29), and embedded in Tissueprep® (Fisher Scientific Inc., Pittsburgh, PA) for sectioning on a rotary microtome. Two were frozen in isopentane cooled by liquid nitrogen, one being freeze-dried and one being sectioned on a cryostat. The last piece was either hand-sectioned with a razor blade and stained with neutral red (2) (in 1978) or processed as described above for sectioning on a rotary microtome (in 1979). Cryostat-prepared sections were stained for diaphorase (32), β-glucosidase (23), esterase (20), peroxidase (20), and phenolase (20) activities. Further details are in reference 16. Cryostat sections also were stained for lipids with Sudan IV, for pectins with ruthenium red, and for tannins with $FeCl_3$ or with the nitroso reaction (20). The $FeCl_3$ solution was mildly basic so as to precipitate the tannins immediately. Hyphae were stained with Sass' hemalum (5). Lignin was detected with phloroglucinol-HCl (20), or using a fluorescence microscope (24) with dark-field illumination (Leitz BG-12 excitation and Kodak Wratten No. 12 barrier filters). Where appropriate, stained cryostat sections were fixed in saline and mounted in Karo corn syrup (20). The studies with cryostat sections concentrated on the first 12 days after inoculation. Paraffin (Tissue-prep) sections from all sampling times were stained in safranin and fast green. Paraffin sections from selected sampling times also were stained with hemalum and erythrosin, phloroglucinol-HCl, Maule's reagents (for lignin, 33), Sudan IV, ruthenium red, and aniline blue (for callose, 20).

Mullick's F-F test (28) was used to detect the formation of a water-impermeable wound periderm induction barrier around the cankers and wounds. Samples were stored on ice for transport to the laboratory and tested beginning the same day. The solutions (2% $FeCl_3$ followed by 4% $K_3Fe[CN]_6$) were allowed to diffuse up the longitudinal axis of the samples for 3 days each.

RESULTS

Lignified zone. In all inoculated hosts, individual hyphae grew out into the surrounding bark tissues. At day 8 (but not at day 5) after inoculation in 1979, in all inoculated hosts, this initial lesion was surrounded by a zone of axial and ray phloem parenchyma and of cortical parenchyma with apparently lignified cell walls (Fig. 1A). In 1978, the apparently lignified zone was observed at day 10 and thereafter. In the NI treatments in 1978 and 1979, lignification was observed at the same times as above, close (5–10 cells) to the wound.

Lignin was indicated by strong staining with phloroglucinol-HCl and by yellowish green fluorescence under ultraviolet light (Fig. 1B and C) of middle lamellae, starting at cell corners. Lignin also was indicated by staining with safranin (Fig. 1A), but not with hematoxylin, Sudan IV, or aniline blue. The lignified zone was negative for Maule's reaction. Stain for β-glucosidase activity in cytoplasm (Fig. 1D) and for peroxidase activity in cell walls was markedly enhanced in the lignifying zone; this also suggested that lignin was being deposited. Stain for phenolase activity in plastids occurred only in the lignifying zone (Fig. 1E and F). Sometimes, cell walls in portions of the lignified zone in the cortex of all host-treatment combinations stained with $FeCl_3$.

The lignified zone ranged in width from 1 to ~15 cells (Figs. 1B and 3B), depending on tissue type and on the orientation of the zone. Occasionally, the cell walls in the lignified zone appeared thickened (Fig. 1F). The zone was translucent in unstained sections when viewed macroscopically or with diffuse epi-illumination. The region of lignification was brown at the cut surface of intact, fresh tissue. In general, for all inoculated hosts, hyphae grew up to the region of lignification but not through it (Fig. 1G), as soon as the zone formed. The hyphae were primarily, but not exclusively, intracellular during the initial stages of infection (Fig. 1G). In all hosts, hyphae tended to colonize the secondary phloem before the cortex. In almost all cases, host cells on the side of the lignified zone nearest the wound were dead at the time of lignification. Cell death was indicated by no staining of vacuoles with neutral red, by no staining of mitochondrialike bodies with diaphorase (Fig. 1H to J), by red staining of nuclei with safranin and fast green, and by the occurrence of plasmolysis (Fig. 1G). Cells on the side of the lignified zone away from the wound were alive, using the obverse of the same criteria (nuclei were grey-green with red nucleoli in safranin and fast green-stained sections; such nuclei are illustrated in Fig. 3B).

With Mullick's F-F test, in 1978, dye diffusion did not stop at the lesion border at day 10 in the S-V, S-H, and S-NI combinations but did in the other combinations. Diffusion stopped in all combinations at day 12. In 1978, the lignified zone was complete at day 10. In 1979, stoppage of dye diffusion first was observed at day 11, and thereafter. In 1979, the lignified zone was complete at day 11.

Initial lesion. The dead, colonized cells surrounded by a lignified zone constituted what is termed the initial lesion. Before and after lignification, hyphae always were surrounded by dead cells. Staining due to basal β-glucosidase (Fig. 1D) and esterase (Fig. 2A) activities in cytoplasm declined rapidly where hyphae were present. Ferric chloride-stainable substances also disappeared in advance of colonizing hyphae in the initial lesion. After colonization, stain for peroxidase activity in parenchymatous cells declined, but less rapidly than the previous substances and activities. Stain for peroxidase activity in phloem fiber walls persisted for long periods (10–15 days) in infected tissue of all hosts, possibly to a greater extent in R trees. In R trees, but not MR or S trees, stain for esterase activity persisted in the middle lamellae and walls of host cells as hyphae colonized them (Fig. 2A).

In the initial lesion, hyphal aggregates of both V and H isolates could be observed in all hosts, but aggregates of long (>50 μm), intercellular hyphae with dense cytoplasm were observed only occasionally in R (Fig. 2B), and MR trees. Aggregates of two (Fig. 2C) to eight (Fig. 2B) densely stained, intercellular hyphae were observed invading, but not penetrating, the lignified zone before mycelial fan formation occurred (before days 18 and 20 in 1978 and 1979, respectively). Before those days in 1978 and 1979, such aggregates of the V isolate were observed in seven of eight cankers on the S trees, but only five of 13 cankers on R (three out of six) and MR (two out of seven) trees (aggregate frequency was not independent of host, $P<0.1$ by a G-test).

TABLE 1. Mean number of hyphae of a virulent and a hypovirulent isolate of *Endothia parasitica* in American and Chinese chestnut lesions between 16 and 30 days after inoculation in 1978

Tree	Fungus[x]	Hyphae per treatment[y] (mean no.)
American		
Stump sprout (S)	V	42 a[z]
	H	34 ab
Large surviving (MR)	V	23 c
	H	26 bc
Chinese (R)	V	28 bc
	H	23 c

[x] V is virulent isolate CR; H is hypovirulent isolate Ep66.

[y] On each canker, the number of hyphae intersecting two anticlinal lines 500 μm long were counted. One line was in the cortex, 500 μm up or down the tree axis from the wound. The second line was in the secondary phloem, about 250 μm below the wound. The ends of the lines were thus about 500 μm apart. There were no mycelial fans in these areas. There were six cankers per treatment, collected at 2- or 3-day intervals from 16 to 30 days after inoculation. Within treatments, each canker was a different age.

[z] Means followed by the same letter are not significantly different according to Duncan's multiple range test. The model used to compute the mean square was a one-way classification with cankers of different ages nested within treatments. Days after inoculation was not included as a covariable because all slopes so estimated were not significantly ($P<0.05$) greater than zero by a t-test.

TABLE 2. Lengths of initial lesions formed after inoculation of American and Chinese chestnut with a virulent (V) and hypovirulent (H) isolate of *Endothia parasitica*

Tree	Fungus[x]	Mean length of all cankers < 21 days after inoculation: 1978 Length[y] (mm)	1978 Observations (no.)	1979 Length[y] (mm)	1979 Observations (no.)
American Stump sprouts (S)	V	12.1 b[z]	6	36.5 a[z]	4
	H	21.1 a	7	32.2 b	4
Large surviving (MR)	V	10.9 b	5	12.5 c	4
	H	12.0 b	5	11.5 c	4
Chinese (R)	V	12.4 b	5	35.5 a	4
	H	18.2 a	6	23.2 b	4

[x] V is virulent isolate CR; H is hypovirulent isolate Ep66.

[y] Each data point is from a separate canker. Within a treatment, replicates were collected on different days, from 10 to 18 (1978) or from 11 to 20 (1979) days after inoculation.

[z] Means followed by the same letter are not significantly different according to Duncan's multiple range test (one-way anova) $P = 0.05$. Days after inoculation was not included as a covariable because all slopes so estimated were not significantly ($P < 0.05$) greater than zero by a t-test.

The numbers of hyphae in the initial lesions increased through day 14, and then remained constant. Between days 16 and 30 in 1978, there were significantly ($P < 0.05$) more hyphae (not including mycelial fans) in the V initial lesions of S trees than in those of R and MR trees (Table 1). The V isolate appeared to colonize infected parts of S trees more extensively than did the H isolate, but no significant ($P < 0.05$) difference was found between isolates (Table 1). These observations could not be made after day 30 because the S-V samples did not include the initial lesion.

The size of the initial lesion was not strongly influenced by host or isolate, but varied between years (Table 2). In other experiments in 1978, 1979, and 1980, it was observed that smaller inoculation holes on American chestnut grafts and seedlings gave smaller initial lesions.

Wound periderm. In all host-treatment combinations, wound periderm formation started next to the lignified zone, at the deepest part of the initial lesion, usually in the secondary phloem, and progressed outward to the outer periderm (Fig. 3A). The phellogen area of all combinations stained intensely with $FeCl_3$. After formation began, it took about 20 days before wound periderm extended to the outer periderm in all treatments on the R and MR trees, and about 30–40 days in the uninoculated S trees (see references 16 or 17 for data sets). These events could not be observed in the inoculated S trees, because wound periderm formation was disrupted by expansion of mycelial fans beyond the

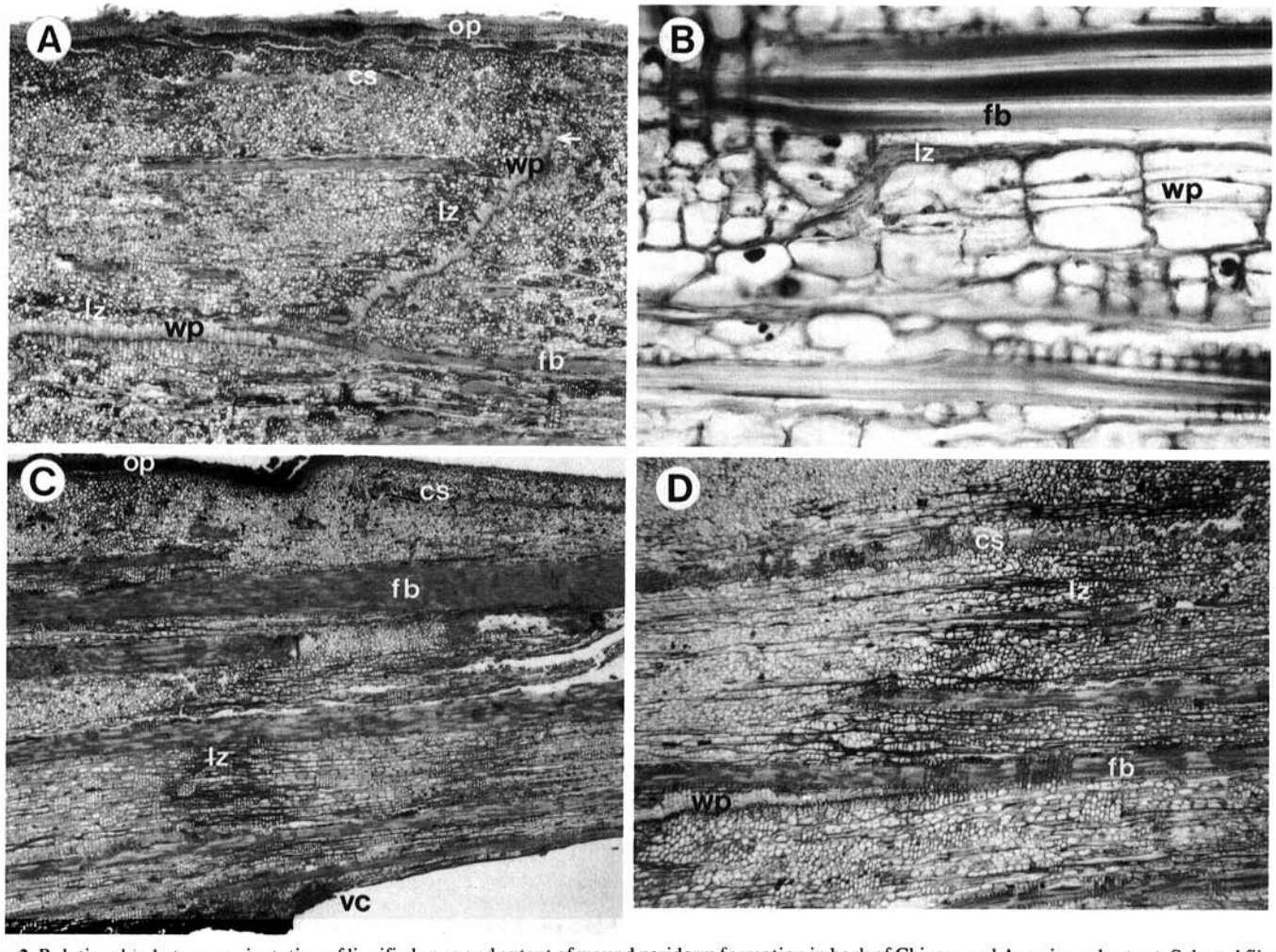

Fig. 3. Relationship between orientation of lignified zone and extent of wound periderm formation in bark of Chinese and American chestnut. Selected fiber bundles (fb) and cortical sclereids (cs) noted to give perspective. Radial sections stained with safranin and fast green. **A,** Day 53. Moderately blight-resistant American chestnut inoculated with a virulent isolate of *Endothia parasitica*. Note wound periderm (wp) next to a lignified zone (lz). Wound periderm is thinner towards outer periderm (op) and does not extend (arrow) to it (×28). **B,** Day 14. Uninoculated blight-susceptible American chestnut stump sprout. Wound periderm formation (wp) had commenced in all uninoculated hosts by this time at interior border of periclinal lignified zones (lz) (×280). **C,** Day 23. Blight-resistant Chinese chestnut cv. Nanking inoculated with virulent isolate. Lignified zone (lz) runs anticlinally from vascular cambium (vc) to outer periderm (op). Note absence of wound periderm (×27). **D,** Day 28. Blight-resistant Chinese chestnut inoculated with hypovirulent isolate. Wound periderm (wp) had not formed where lignified zone (lz) turns anticlinally to reach the outer periderm above the cortical sclereids (cs) (×36).

initial lesion. In all host-treatment combinations, after formation started at one location, it took 4–8 days for phellem to form at that location and 8 days after that for the number of phellem cell layers to reach a maximum. There were approximately nine phellem and seven phelloderm cell layers in the finished wound periderm of all treatments in the R and MR trees and seven and four in the S-NI combination (Table 3). There were significantly fewer phellem cell layers in the S-V and S-H combinations than in the other combinations.

In 1978, wound periderm first was observed (Fig. 3B) at day 12 in six of the nine combinations. The three exceptions were R-V, where periderm first was observed at day 10, and S-V and S-H, days 14 and 23, respectively. In 1979, wound periderm first was observed at day 14 in five of the nine combinations. The four exceptions were R-NI and MR-H, day 11, and S-V and S-H, days 20 and 48, respectively (no samples were collected between days 20 and 48 in 1979). In a preliminary experiment in 1977, with sampling every 2 days, wound periderm first was observed at day 16 in all host-treatment combinations.

When the lignified zone was oriented completely anticlinally, initiation of wound periderm formation in any host was greatly delayed compared to sites in which the lignified zone had periclinal orientation (Fig. 3C). In 1978, in the secondary phloem of all uninoculated hosts, wound periderm next to anticlinal portions of lignified zones first was observed at day 23, as compared to day 12 for periclinal portions. The *rates* of addition of phellem and phelloderm cells to anticlinal periderms were very similar to the rates for periclinal periderms (these observations could not be made for the inoculated treatments because expanding mycelial fans killed the phellogen in the S trees). In all host-treatment combinations, there were occasions beyond day 23 when no wound periderm existed next to anticlinal portions of lignified zones. At points where a lignified zone turned from a periclinal to an anticlinal orientation, the number of cell layers in wound periderm of all hosts decreased (Fig. 3D).

The higher frequency of occurrence of delayed initiation of wound periderm in inoculated S trees was associated with their higher frequency of completely anticlinal lignified zones. When the lignified zone in inoculated S trees had periclinal portions, wound periderm initiation was not delayed in comparison to the other host-treatment combinations. In 1978, between days 13 and 21, five of 13 S tree cankers versus one of 20 (the one canker being from the R-V combination) R and MR cankers had completely anticlinal lignified zones (significantly different [$P < 0.05$] by a G-test). Observation of lignified zones in S trees after day 21 was difficult or impossible due to mycelial fan formation and growth. Infection to the vascular cambium commonly led to completely anticlinal lignified zones, whereas initial lesions not reaching the vascular cambium had large periclinal inner margins, since most lesions were 1–3 cm long (Table 2). All the initial lesions in S trees reached the vascular cambium, but most (>60%) in MR and R trees did not, probably because their barks were significantly ($P < 0.04$, by an F-test) thicker (3.0 mm for both the MR and the R trees) than the bark of the S trees (2.0 mm). In 1978 and 1979, the initial lesions on R and MR trees averaged 0.86 ± 0.28 mm in depth (plus 1 mm for the inoculation wound) when they did not reach the vascular cambium.

The thinner bark of the S trees was related to their smaller diameter (thickness [mm] = 1.09 + 0.285 dbh [cm], $R^2 = 0.37$ for 66 samples, both coefficients significantly [$P < 0.001$] different from zero). The inoculated portions of the S trees ranged from 2–3 cm in diameter, and those of the MR and R trees ranged from 3–6 cm. The S trees were shaded by a forest overstory, whereas the R and MR trees were only partially shaded, and this shading probably kept the S trees small (16). Shading of the S trees also was associated with the presence of rhytidome, which is not found on similar-sized S trees growing in full sun (16). Rhytidome on S trees may have delayed the time it took wound periderm to extend to the outer periderm in comparison to the R and MR tree branches, which had less rhytidome. In any sample with rhytidome, wound periderm tended to extend around the stem before connecting with the most recent secondary periderm. In some instances, no connection occurred.

Mycelial fans. Mycelial fans (Fig. 4A) were observed in all hosts. All fans grew intercellularly. Ruthenium red staining indicated presence of pectin in the middle lamellae of host cells in the region of the fan tip, but the middle lamellae there swelled (Fig. 4B). Physical deformation of bark tissue by fans and splitting of tissue in front of fans (Fig. 4A) were observed commonly. The walls of a few host cells near the tips of fans were fragmented, apparently by hyphae proliferating in their lumens. When fans grew between phloem fiber bundles, the nonlignified, interfascicular cells often were pushed to one side and crushed, as indicated by a continuity between crushed and uncrushed host cells at points where fan thickness lessened. Where fans penetrated lignified zones, the host cells were not crushed but were split physically; the host cells on either side of the fan in Fig. 5B fit together perfectly before the fan intruded.

TABLE 3. Mean number of cell layers in phellem and phelloderm of wound periderm in American and Chinese chestnut between 30 and 90 days after inoculation in 1978 with a virulent (V) and a hypovirulent (H) isolate of *Endothia parasitica*

Tree	Fungus[w]	Cell layers (mean no.)[x]: In phellem[y]	Cell layers (mean no.)[x]: In phelloderm[y]	Observations (no.)
American				
Stump sprout (S)	V	2.0 c[y]	1.3 c[y]	14
	H	3.9 c	2.8 bc	13
	none	6.9 b	3.5 b	12
Large surviving (MR)	V	9.3 ab	6.8 a	8
	H	9.0 ab	6.3 ab	3
	none	9.0 ab	8.0 a	6
Chinese (R)	V	9.9 a	6.9 a	12
	H	9.4 ab	6.1 ab	12[z]
	none	9.5 ab	5.3 ab	12

[w] V is the virulent isolate, CR; H is the hypovirulent isolate, Ep66.

[x] Each observation was an average of the number of cell layers in the phellem or phelloderm of wound periderm in the cortex and secondary phloem, replicated over treatments of different age. Observations in the cortical sclereids and outer periderm were not included because there were too many missing values due to presence of rhytidome and tearing of sections.

[y] Means followed by the same letter are not significantly different according to Duncan's multiple range test, $P = 0.05$. A two-way anova was run using treatment (tree and isolate) and location in bark (secondary phloem or cortex) as the classification variables. The interaction terms were not significant. Days after inoculation was not included as a covariable, because when the slopes were estimated, only those for the Chinese chestnut with either the V isolate or with no inoculum were significantly ($P < 0.05$) greater than zero. The slopes for these two treatments were skewed upward by one high value for one canker of each at 65 days after inoculation.

[z] There were 13 observations for the phellem.

Hyphae at the tips of fans were densely cytoplasmic (Fig. 4B), but hyphae 1 mm behind the tips were vacuolate (Fig. 4D), leading to a higher affinity for cytoplasmic stains of the tip compared to the rest of the fan (Fig. 4C). Mycelial fans are termed advancing if no lignification or wound periderm formation occurred *in front* of them and they had densely stained hyphae at their tips. Neutral red staining indicated death of parenchyma cells at least 350 μm in front of advancing mycelial fans. Death also was indicated by safranin staining of parenchyma (Fig. 4C), the occurrence of plasmolysis, and the appearance of nuclei in safranin, fast green-stained sections. Cells underneath and above fans died if the cells were not insulated from the fans by lignified zones or wound periderm. This host cell death probably was the reason the wound periderm in the S-V and S-H combinations had significantly fewer cell layers (Table 3). No hyphae were observed in front of advancing fans.

In the S-V host-treatment combination in 1978, mycelial fans first were observed at day 18. Some possible stages leading to fan formation are shown in Fig. 2C and B. Mycelial fans of the V isolate in S trees had penetrated the lignified zone when they first were observed at day 18. With the H isolate on S trees, mycelial fans first were observed at day 16 in 1978, but these remained behind the lignified zone until day 28. In 1979, no fans of the V isolate were observed during the first 20 days on S trees. Fans of the H isolate, located behind the lignified zone, first were observed at day 17 in S trees, but no fans were observed at day 20. In S trees between days 18 and 79, 32 fans of the V isolate were significantly ($P<0.034$ by a t-test) thicker (83 μm) at their thickest point (near the tip) in longitudinal section than 33 fans of the H isolate (56 μm). In a separate experiment on S trees, fans that had expanded beyond the initial lesion were observed in cankers of three hypovirulent isolates. Fans were not observed in cankers of two other hypovirulent isolates which had not expanded beyond the initial lesion. In older cankers of Ep66, but not other H nor V isolates, hyphae 1 mm behind the fan tip appeared lysed.

Mycelial fans of the H isolate were not observed in cankers on MR and R trees. Advancing mycelial fans of the V isolate were observed in the MR tree only at day 72 in 1978, and at days 46 and 81 in 1979. Advancing mycelial fans of the V isolate were observed in R trees at days 46 and 81 in 1979, but not at all in 1978. These fans in MR and R trees had penetrated the lignified zone, where wound periderm had not formed (wound periderm gap), although one, in the MR tree, also breached a wound periderm (Fig. 5A and B) with two or more phellem cell layers. No other penetrations of the lignified zone or wound periderm of the initial lesion were observed in MR and R trees. Typically, only one fan, located in the outer bark layers, was observed in the two resistant hosts (Fig. 4C), whereas multiple fans (Fig. 4A), with one often at the vascular cambium, were observed commonly in S trees. In the S trees, the leading fan most frequently was in the middle bark depths (Fig.

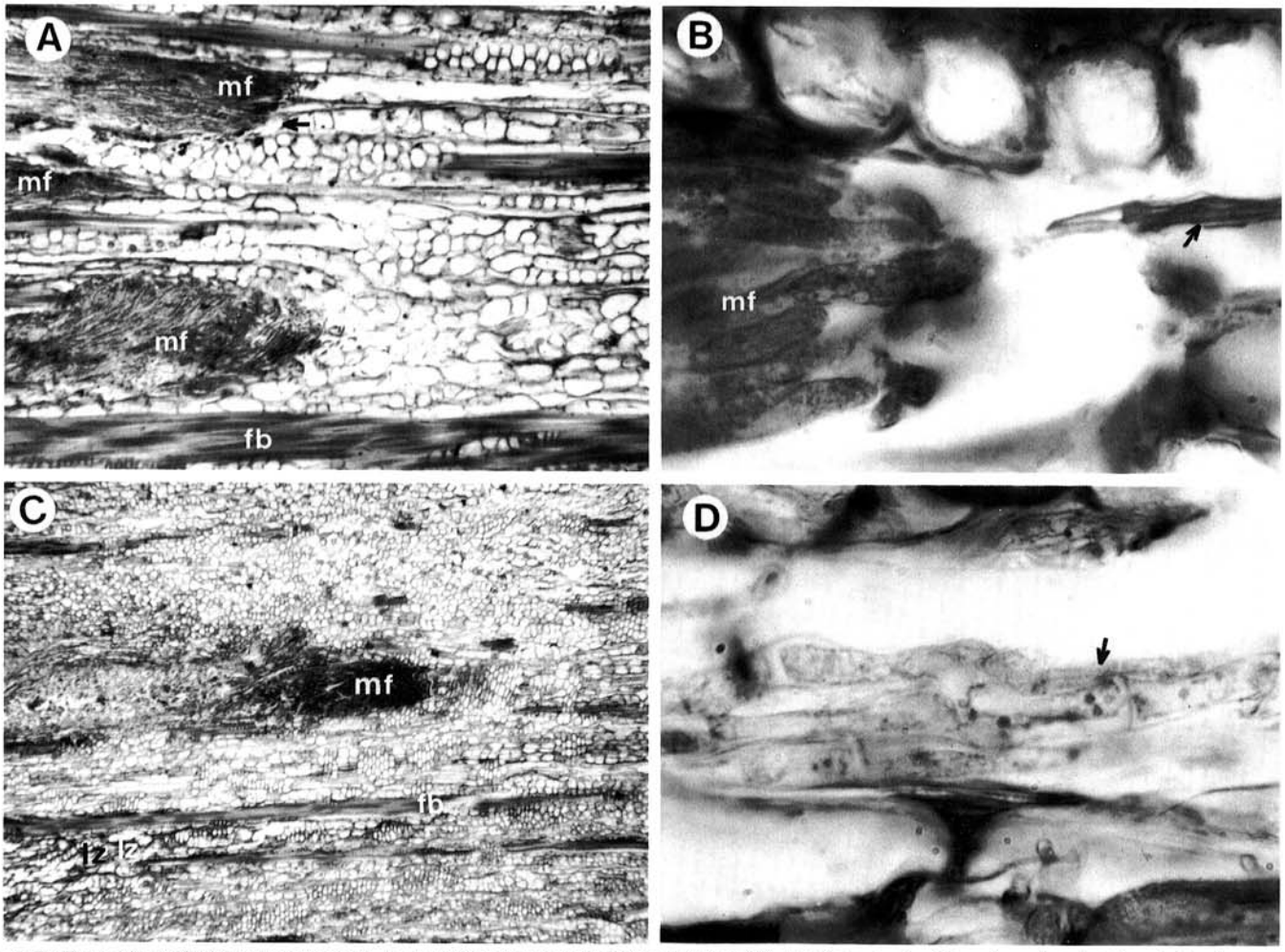

Fig. 4. Radial sections of blight-susceptible **(A, B, and D)** and moderately blight-resistant **(C)** American chestnut trees inoculated with a virulent isolate of *Endothia parasitica* showing appearance of mycelial fans. Sections stained with safranin and fast green **(A and C)** or hematoxylin and erythrosin **(B and D)**. Selected fiber bundles (fb) noted to give perspective. **A,** Three mycelial fans (mf) at day 23. Mycelial fans outside the lignified zone were evident by day 18. Arrow points to cells bent and crushed by fan (×100). **B,** Day 21. Tip of mycelial fan, illustrating dense cytoplasm at tip. Note apparent swelling of middle lamella in advance of hyphae (arrow) (×1,070). **C,** Day 72. Mycelial fan (mf) (and associated lignified zone [lz]) observed in this host only in this sample in 1978. Note dark staining (safranin) cells in front of fan, indicating cell death. Only tip of fan stains darkly with safranin (×35). **D,** Hyphae of mycelial fan of B approximately 2 mm behind tip of fan, illustrating vacuolated appearance of hyphae away from tip of fan. Compare **B and D** with **C**. Note double nuclei (arrow). Hyphae like these eventually lysed in the hypovirulent isolate, Ep66 (×1,040).

4A). In every observed instance of canker enlargement, a mycelial fan occurred at the canker margin.

Canker growth. The S-V combination showed a significantly ($P < 0.05$) higher rate of canker growth (slope of canker length versus time) than other combinations in 1978 (Table 4). There were not enough observations to make these calculations for the 1979 experiment. In 1978, the slope for the S-H combination was intermediate between that of S-V and those of MR-V and MR-H, and was significantly larger than the slopes for R-V and R-H. The main effect for the H treatment slopes was significantly ($P < 0.001$, by an F-test) lower than the V treatment slopes. In a separate experiment in 1979, the lengths of 24 cankers incited by the V isolate on S trees were measured every 2–4 days from 6 to 42 days after inoculation. The initial lesion formed in 8–11 days and no further canker expression occurred until days 24–26. At days 24–26, elongation of the 24 cankers started again and continued, basically at a constant rate of about 0.85 mm/day, until the trees were encircled, or until low temperatures halted the process. In the histopathology experiments, apparent cessation of initial lesion expansion occurred concurrently with the onset of lignification, around day 10 in 1978 and day 8 in 1979; canker elongation in the S-V combination resumed about day 18 in 1978, when mycelial fan formation and expansion began.

DISCUSSION

These results indicate that the rate and extent of mycelial fan formation are the most important factors determining the enlargement of chestnut blight cankers. When V canker expansion occurred on blight-resistant *C. dentata* (MR) and *C. mollissima* (R), generally only one mycelial fan formed, located in superficial bark layers and underlain by a lignified zone and wound periderm. These fans formed later and did not grow in length and width as rapidly as fans in susceptible *C. dentata* (S), in which multiple fans, with one often at the vascular cambium, were common. In the R and MR trees, lack of enlargement of cankers was associated with lack of mycelial fans of both V and H isolates of *E. parasitica*; the same held in the S trees for some isolates with cytoplasmic hypovirulence.

Physical pressure probably is involved in the advance of mycelial fans, as has been observed in other systems (8,34). The lignified zone was breached by fans but not by individual hyphae or aggregates of two to eight hyphae; no fans of *E. parasitica* unequivocably penetrated *fully formed* wound periderm. Rather, they appeared to grow through gaps in wound periderm in most instances. Microscopical observation indicated that the gelatinous zone commonly reported (apparently from macroscopic observation only) to exist immediately in advance of fans (25) is composed of the densely stainable hyphae at the edge of the fan.

TABLE 4. Slope of canker length on American and Chinese chestnut versus days after inoculation in 1978 with a virulent and hypovirulent isolate of *Endothia parasitica*

Tree	Fungus[x]	Slope of canker length[y] (mm/day)	Observations (no.)
American			
Stump sprout (S)	V	0.754 a[z]	20
	H	0.484 b	21
Large surviving (MR)	V	0.350 bc	8
	H	0.301 bc	9
Chinese (R)	V	0.189 c	18
	H	0.038 c	18

[x] V is virulent isolate, CR; H is hypovirulent isolate, Ep66.
[y] Each data point was from a separate canker. Points were collected between 10 and 86 days after inoculation.
[z] Slopes followed by the same letter are not significantly different according to Duncan's multiple range test, $P = 0.05$, after analysis of covariance.

The susceptibility of American chestnut to blight did not appear to involve deficiencies in the rate or extent of wound periderm formation. Except as noted below, neither did V, as opposed to H, *E. parasitica* appear to induce such deficiencies, as has been claimed for European chestnut (15). Rates of formation of the lignified zone, a wound periderm induction barrier, were similar in all host-treatment combinations, as were rates of addition of phellem and phelloderm cell layers at one location, once formation began. We did observe delayed extension of wound periderm to the outer periderm in the S-NI combination, but this may have been due to the propensity of S trees to form rhytidome. Lack of connection between successive periderms has been observed in other plants (13, pages 248-250). This suggests that presence of rhytidome may increase the size and frequency of occurrence of superficial cankers. The greater frequency of canker expansion on the MR and R trees in 1979 than in 1978 indicates that environmental factors can affect the size of superficial cankers.

When the initial lesions did not reach the vascular cambium, the deepest parts of the lesion were bordered by a periclinal lignified zone; wound periderm formation began there and spread outwards. Wound periderm formation in any host-treatment combination was delayed next to anticlinal lignified zones, such as

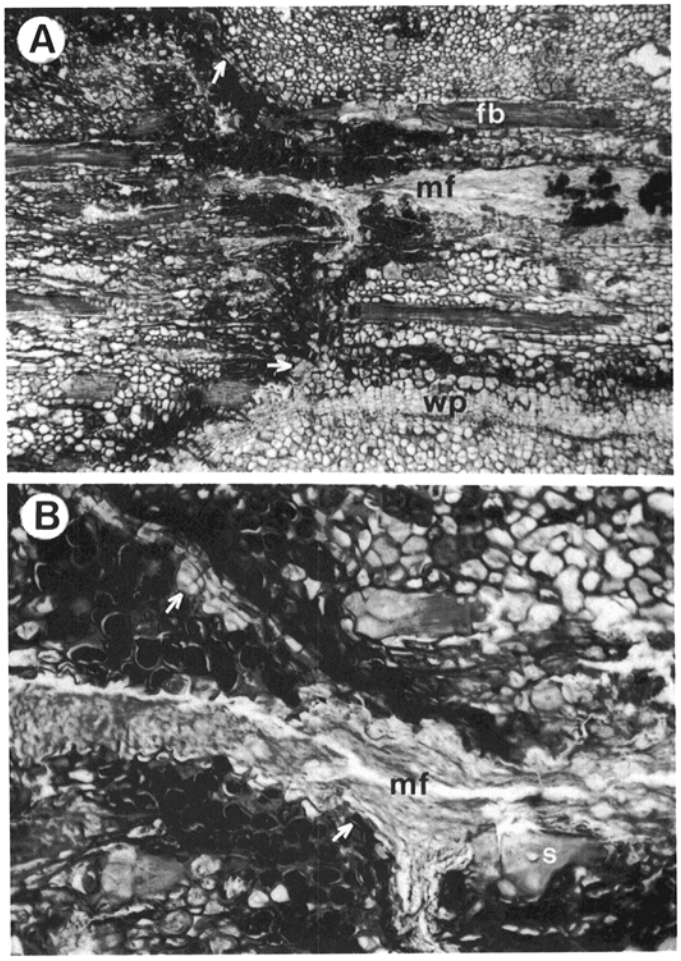

Fig. 5. Moderately blight-resistant American chestnut inoculated with virulent isolate of *Endothia parasitica*, showing penetration of a wound periderm with two or more phellem cell layers (arrows) by a mycelial fan (mf). The older infected tissue is to the left. Day 81, 1979. Selected fiber bundles (fb) and sclereids (s) are labeled. **A,** A new wound periderm (wp) has formed, branching off from the old one below the lower arrow, separating the fan (mf) and hyphae from uninfected tissue below it. The breached wound periderm is also evident at the upper arrow (×43). **B,** Detail of canker in **A,** showing wound periderm (upper arrow) extending down to point where it was breached by a mycelial fan. The wound periderm continued at the lower arrow, but this is not evident in the photograph. There was a gap in the wound periderm at this point in 5% of the sections of this canker (×170).

where the zones joined the outer periderm, but not next to periclinal lignified zones. This pattern of wound periderm formation is probably the principle cause of the superficial location of slow-growing mycelial fans of V isolates in resistant trees or of H isolates in susceptible trees. The environmentally induced thin bark of the S trees led to infection to the vascular cambium after artificial inoculation. This resulted in a higher frequency of occurrence of anticlinal lignified zones in S trees compared to MR and R trees. Thus, wound periderm formation was initiated later in S trees than MR and R trees, in some instances. There was a higher frequency of occurrence *at* the lignified zone of small aggregates of densely stained hyphae in S trees than MR and R trees. This may have contributed to delayed initiation of wound periderm. These hyphal aggregates probably were precursors of mycelial fans. Once fans expanded past the lignified zone, death of phellogen cells halted wound periderm formation.

The variation in initial lesion size for V versus H isolates between 1978 and 1979 indicates that initial lesions must be evaluated carefully in blight resistance and hypovirulence determinations and in studies concerned with the early stages of pathogen growth and canker development. Only early stages of pathogenesis may occur on 1-yr-old seedlings. The nearly identical sizes of initial lesions in R, MR, and S trees resemble the similar rate of colonization by *E. parasitica* of tissue cultures from resistant and susceptible chestnut (18). Hebard (*unpublished*) observed intense sporulation by *E. parasitica* on tissue cultures from susceptible but not resistant chestnut; the difference occurred 1 mo after inoculation, after the tissue cultures were completely colonized. This may parallel mycelial fan formation in the intact plant.

A key to blight susceptibility seems to be the ability of *E. parasitica* to obtain nutrients from, and not to be inhibited by, dying and dead chestnut cells while forming mycelial fans. Differences in preformed carbon substrates (12,18,31), especially tannins, between susceptible and resistant chestnut may be involved in the greater growth of *E. parasitica* in susceptible plants. Elkins et al (12) found that dry weights of *E. parasitica* after growth on an aqueous extract of milled air-dried bark were higher for S than for R trees. Additionally, a higher percentage of tannins was utilized in the S tree extract. We found that cell wall-localized esterase activity persisted after infection only in R trees. The esterase might be involved in the complexing of tannins with proteins and carbohydrates, rendering the complex unavailable as nutrients to *E. parasitica* (1). McCarroll (25) found that inner bark of *C. mollissima*, compared to *C. dentata*, turned brown when treated with low concentrations of buffered oxalate or various organic acids. Oxalic acid is a presumed toxin of *E. parasitica*. McCarroll (25) also found that inhibitors extracted from R trees were more inhibitory than those from S trees against the endopolygalacturonases of *E. parasitica*.

The histochemical procedures for detecting lignin are not definitive. In particular, use of fluorescence microscopy for this purpose apparently has not been explored systematically. Mayama and Shishiyama (24) reported that lignified tissues in barley fluoresced yellowish green. In the present study, cell walls of the putative lignified zone, or wound periderm induction barrier, fluoresced yellowish green and stained with phloroglucinol-HCl, as did those in all sclerenchyma and in outer phellem layers of the outer periderm. Only the walls of the latter two cell types also stained with Maule's reagents (33). Craft and Audia (10) and Delon (11) previously reported that phenolase and peroxidase activities are enhanced in developing wound periderm induction barriers. No such previous report for β-glucosidase activity exists. The enhanced β-glucosidase and phenolase activities may play a role in the formation of phytoalexins either in addition to or rather than in lignification. There was no evidence that coupling of tannins or quinones with cell walls gave the lignin reactions we observed; such coupling, indicated by $FeCl_3$ staining of cell walls, was observed only in scattered cortical cells near or in the lignified zone.

There are reports indicating that wound periderm induction barriers are lignified (9,36) or not suberized (28). The reports of suberization of wound periderm induction barriers are based on staining with ammoniacal crystal violet, which stains lignin also (26). However, confusion between "suberization" to form induction barriers and suberization of wound periderm phellem appears to persist (22). Lignification such as in Fig. 4C has been reported previously in American chestnut bark near mycelial fans of *E. parasitica* (7,21). Uchida (35) observed a wound periderm induction barrier in Japanese chestnut, but did not test for lignin. The results with Mullick's (28) F-F test support the concept that a lignified wound periderm induction barrier is water impermeable.

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