Virulence of *Endothia parasitica* Isolated from Surviving American Chestnut Trees

Gary J. Griffin

Virginia Polytechnic Institute and State University

John R. Elkins

Bluefield State College, Bluefield, WV 24701

Gail Tomimatsu and Frederick V. Hebard

Virginia Polytechnic Institute and State University, Blacksburg, VA 24061

ABSTRACT.— Large surviving American chestnut trees may possess blight resistance if they are not infected with American hypovirulent strains of *Endothia parasitica.* To investigate this, isolates of *E. parasitica* were obtained in 1975, 1976 and 1977 from cankered tissues of large, surviving and small American chestnut trees (*Castanea dentata),* and from cankered Chinese chestnut trees (*C. mollissima)* growing in the Appalachian region. These tissue isolates and single-conidium progeny of tissue isolates were examined for pigment production, linear growth rate *in vitro,* tannin utilization and pathogenicity on disease-free American chestnut stump sprouts. A large variation in pathogenicity among isolates was observed. Most isolates from surviving American chestnut trees were moderately or highly pathogenic, when comparisons were made to two isolates from blight-killed American chestnut trees and a European-derived hypovirulent isolate, Ep43. Some isolates from a large American chestnut tree in Virginia were hypovirulent (weakly pathogenic).

INTRODUCTION

The American chestnut (*Castanea dentata* [Marsh.] Borkh.) grew to be a large tree before *Endothia parasitica* (Murr.) P. J. & H. W. And. devastated the extensive natural stands in the Appalachian region. Today, only a few large trees (Fig. 1) remain in scattered locations throughout the region. Typically they are extensively infected with *E. parasitica* and show various degrees of dieback in the crown and bole due to this infection. American stump sprouts have become established since the blight but show similar or greater levels of disease. The residual trees may possess some degree of blight resistance if they are not infected with American hypovirulent strains of *E. parasitica.* This investigation was undertaken to examine this question for selected surviving American chestnut trees in Virginia, West Virginia, and other states within the natural range of the American chestnut.

Figure 1. Surviving American chestnut tree in West Virginia from which *Endothia parasitica* was isolated for use in pathogenicity studies.

METHODS AND MATERIALS

In 1975, 1976 and 1977, bark samples were obtained at the canker margins of surviving American and Chinese chestnut (C. *mollissima* Bl.) trees. Samples were placed in plastic bags before tissue pieces were transported to the laboratory and plated on acidified Difco potato dextrose agar (APDA). *Endothia parasitica* isolates growing from these tissue pieces (termed "tissue isolates") were trans-

ferred to PDA slants; mature cultures of these tissue isolates were stored, if necessary, under mineral oil until used in pathogenicity tests. Single-conidium isolates were obtained from mature tissue isolates soon after initial isolations were performed. Tests made on approximately 150 conidia of two representative isolates with Feulgen stain (Johansen, 1940) indicated that most conidia were uninucleate, but that some conidia (10-20 percent) contained two bodies, often close together, that stained.

Degree of pigmentation and linear growth measurements of 20 single-conidium isolates, for each of several tissue isolates, were determined at room temperature (26-28 C) and in room light (cool-white fluorescent) after 6 and 12 days incubation. Tissue isolates, together with the darkest pigmented and the lightest pigmented single-conidium isolates, were selected for pathogenicity trials. Most tissue isolates (W, AP, C, WE, SW, MC, IV, KE, RE, PL) were obtained from American chestnut trees with a diameter breast height of 27-102 cm; other isolates (D, AR, CH, CK, ALA) were obtained from American chestnut trees with a diameter breast height of 10-22 cm. Isolates CCS, HF20 and HF38 were obtained from Chinese chestnut. European-derived (Van Alfen *et al.,* 1975) hypovirulent isolate, Ep43 (obtained from R. A. Jaynes, Conn. Agr. Exp. Sta., New Haven), was used as a reference isolate in these trials. In addition, two *E. parasitica* isolates, CR and PC (Table 1), from blight-killed American chestnut trees were used for reference. Blight-free American chestnut stump sprouts (circa 2 to 4 cm diameter breast height) growing in the Jefferson National Forest near Blacksburg, Virginia, were used for pathogenicity tests. The cork-borer-agar-disc method of inoculation was used. Five cork-borer wounds (0.7 cm diameter), approximately 50 cm apart, were made to the vascular cambium for each tree. Potato-dextrose-agar discs of five different *E. parasitica* isolates were inserted into the wounds on each tree, using aseptic technique. Inoculated wounds were covered with masking tape. Five replicate inoculations, on different trees, were made for each tissue isolate or single-conidium isolate. In 1976 and 1977, inoculations were made on June 1 and May 23, respectively; canker lengths were determined on August 14 and October 1 in 1976 and on August 3 and October 11 in 1977. Pathogenicity ratings of isolates were based on canker length measurements made at both time intervals. However, emphasis was placed on August measurements as some cankers were not measurable in October due to brown staining of the bark or to tree death.

Utilization of American chestnut tannins by *E. parasitica* isolates was determined by inoculating 10 ml of aqueous bark extract (1 g powdered young-shoot bark/10 ml water) in a 20-ml screw-cap vial, with 10^6 conidia. Tannin assays were performed by the method of Elkins *et al.* (1977) on 0.5 ml subsamples removed from each of two vials per isolate after 17 days incubation at room temperature (21 C).

RESULTS AND DISCUSSION

Pigmentation and linear growth. All tissue isolates produced yellow-orange pigmented colonies on PDA, typical of *E. parasitica* (Roane and Stipes, 1976; Shear *et al.,* 1917), although there were often differences in colony texture, pigment intensity, pigment distribution in the colony and the time required for pigment production. Single-conidium progeny of almost all tissue isolates also varied to some degree in the intensity of pigmentation produced, as well as in the amount of linear growth produced after six days incubation (Table 1). Two tissue isolates (D and AR), as well as some single-conidium progeny of these isolates (Table 1), produced pigment at a much later stage in colony growth than other *E. parasitica* isolates. Unlike white Ep43 isolates, however, all D and AR isolates were pigmented after 12 days incubation in room light (Table 1). Both white and yellow-orange (normal) single-conidium progeny types were obtained from white Ep43 as has been found for white European hypovirulent strains (Bonifacio and Turchetti, 1973; Grente and Sauret, 1969). However, in separate tests, light influenced the frequency of pigmented colonies and the rate of pigment formation by single-conidium progeny of white (at six days) Ep43. Some tests were conducted at 25 C in a Sherer-Gillette Model CEL-27-14 growth chamber in continuous darkness or continuous light (2,000 ft-c) supplied by cool-white fluorescent lamps. Other tests were conducted in the laboratory under room light. After six days of incubation, 14 of 23 dark-incubated monoconidial progeny on Difco PDA did not contain pigment, while only 1 of 23 light-incubated (growth chamber) progeny did not contain pigment (were entirely white). Two of 20 room-light-incubated progeny were entirely white. After 12 days of incubation, 2 of 23 dark-incubated progeny were entirely white. In contrast, no light-incubated (growth chamber) progeny were entirely white, and 1 of 20 room-light-incubated progeny was entirely white. At this time, light-incubated (growth chamber) progeny exhibited greater intensities of yellow-orange pigment than progeny from the other two treatments. Although no information on pigmentation was given, Barnett and Lilly (1952) observed greater production of pycnidia by *E. parasitica* under conditions of continuous light or alternating light and dark than under continuous darkness.

Virulence. Among 20 isolates of *E. parasitica* examined in 1976 (Fig. 2) and 25 isolates examined in 1977 (Figs. 3 and 4), a wide range in pathogenicity was observed. Some isolates from surviving American chestnut trees were as pathogenic as the two reference "killer" isolates; most isolates were moderately or highly pathogenic. No relation of linear growth *in vitro* or degree of pigmentation to

Table 1
Source, colony color and linear growth of single-conidium progeny of *Endothia parasitica* isolates used in pathogenicity tests on American chestnut.

Tissue or single-conidium isolate	State	Chestnut source	Linear growth range 6 days	Linear growth range 12 days	Color range 6 days
			cm	*cm*	
ALA	NY	Amer.	6.8 - 7.4[a]	8.7[ab]	2[ac]
AP	VA	Amer.	4.7 - 7.3	8.7[b]	4-5
AR	VA	Amer.	1.5 - 4.8	3.3 - 8.7[b]	0-3
C	VA	Amer.	4.4 - 6.0	7.7 - 8.7[b]	3
CCS	WV	Chin.	4.8 - 5.9	8.7[b]	2-4
CH	VA	Amer.	4.2 - 5.1	8.7[b]	3
CK	TN	Amer.	2.5 - 5.0	7.6 - 8.7[b]	1-4
CR	WV	Amer.	-[d]	-[d]	-[d]
D	VA	Amer.	2.3 - 6.3	-[d]	0-4
D13[f]	VA	Amer.	4.0 - 5.1	7.1 - 8.7[b]	4
D19[f]	VA	Amer.	4.0 - 4.8	6.6 - 7.7	4
Ep43	—	—	2.9 - 5.2	8.7[b]	0-4[e]
HF20	VA	Chin.	5.0 - 5.6	8.7[b]	2-3
HF38	VA	Chin.	3.4 - 4.2	8.7[b]	2-4
IV	NH	Amer.	6.3 - 7.2	8.7[b]	2-3
KE	PA	Amer.	3.9 - 5.3	6.0 - 8.7[b]	2-4
MC	WV	Amer.	3.2 - 4.8	8.7[b]	2-3
PC	VA	Amer.	3.4 - 4.7	6.5 - 8.7[b]	2-3
PCM[f]	VA	Amer.	2.9 - 4.1	7.2 - 8.7[b]	2
PL	ME	Amer.	3.5 - 4.5	8.7[b]	2-3
RE	WV	Amer.	3.9 - 5.5	5.5 - 7.8	3
SW	WV	Amer.	4.0 - 7.1	4.6 - 8.7[b]	2-4
W	VA	Amer.	3.9 - 5.8	8.7[b]	2-4
W8[f]	VA	Amer.	4.1 - 5.3	7.7 - 8.7[b]	2-4
W20[f]	VA	Amer.	3.9 - 4.9	8.7[b]	2-3
WE	WV	Amer.	6.0 - 6.9	6.5 - 8.7[b]	2-4

[a] Based on 20 single-conidium colonies for each isolate grown in room light on Difco PDA.

[b] Growth limited by diameter of the petri plate.

[c] Color scale: 0 = white, 1 = very light yellow-orange, 2 = light yellow-orange, 3 = yellow-orange, 4 = dark-yellow-orange, 5 = very dark-yellow-orange.

[d] No determination.

[e] Some isolates still white after 12 days incubation.

[f] PCM is a colony sector from tissue isolate PC; W20, W8, D13 and D19 are single conidium isolates from tissue isolates W and D.

pathogenicity was observed. In 1976, one isolate, W-20, was hypovirulent (weakly pathogenic) and produced a mean canker length smaller than that produced by the European-derived hypovirulent Ep43 after 2.5 months; canker length produced by W-20 was slightly greater after 4 months than canker length produced by Ep43. This isolate was a dark-yellow-orange single-conidium isolate derived from a tissue isolate, W, that was highly pathogenic (Fig. 2). Other single-conidium isolates of W were not hypovirulent, nor was a single-conidium isolate, W-20-1, derived from W-20. The latter isolate and other representative isolates of *E. parasitica*, examined for pathogenicity in 1976, utilized 4 to 16 percent of the tannins from aqueous extracts of American chestnut bark, but no relationship of tannin utilization to pathogenicity was apparent (Table 2).

To confirm the hypovirulence of the American W-20, a second experiment was performed in 1977. In December, 1976, bark tissues of cankers were collected from trees inoculated with W, W-20 and Ep43 isolates. All bark tissues of trees inoculated with Ep43 yielded only white cultural forms of the

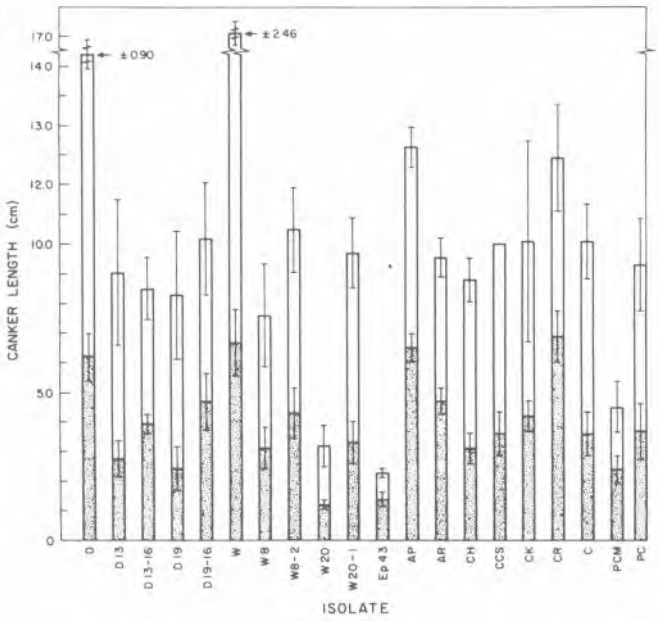

Figure 2. Canker lengths after 2.5 months(stippled bar) and 4 months (total bar) produced by *Endothia parasitica* isolates on stump sprouts of American chestnut in 1976. Variation is indicated as standard error.

fungus, although a yellow-orange sector developed in one colony as it grew from bark tissue on APDA. All colonies of W and W-20 were yellow-orange; both of these isolates were single-spored from PDA cultures, and dark- and light-pigmented progeny (W-2 [77], W-19 [77], W-20-21 and W-20-19) were selected for pathogenicity trials along with white Ep43 (WEp43) and the yellow-orange Ep43 (YEp43) obtained from the sector. In these trials, only the light-pigmented W-2(77) and W were hypovirulent 2.5 and 4.5 months after inoculation (Fig. 3); WEp43 was moderately pathogenic in the early part of the growing season, but little or no canker extension occurred after the August measurements. This restricted growth in the latter part of the growing season was not observed for any of the other isolates. Isolates W-20-19, W-20-21, W-20176) and W-19(77) were moderately pathogenic, while YEp43 was virulent (highly pathogenic). Findings of Bonifacio and Turchetti (1973) and Grente and Sauret (1969) indicated that some single-conidium progeny of European hypovirulent isolates may be virulent. Bark tissue isolation attempts, made in October, 1977, indicated that both yellow-orange and white forms of Ep43 were present in WEp43-inoculated trees; 17 of 30 tissue platings (six for each of five trees) yielded yellow-orange isolates, while 10 of 30 were white. These results indicate that some instability of hypovirulence occurred for WEp43 and W-20 in 1977, and confirm the ability of W to yield hypovirulent single-conidium progeny. That W was hypovirulent in 1977, but not in 1976, suggests that some macromolecular changes occurred during the winter months in the thallus of this isolate.

Table 2
Tannin utilization from aqueous extracts of American chestnut bark by isolates of *Endothia parasitica*.

Isolate	Percent Tannin change	Pathogenicity
W	-9.0[a]	high
W-20[b]	-12.6	low
AP	-16.2	high
CR-1[b]	-6.3	high
CR-2[b]	-7.2	high
CCS	-3.6	moderate
PC	-16.2	moderate
PCM	-10.8	moderate
Ep43	-8.1	low

[a] Represents the decrease in tannin concentration in aqueous extracts of American chestnut bark after 17 days incubation at 21 C.
[b] W-20 is a single-conidium isolate from tissue isolate W, and CR-1 and CR-2 are replicates of tissue isolate CR.

In 1977 tests, other tissue isolates of *E. parasitica* from surviving American chestnut trees, single-conidium progeny of these tissue isolates, and tissue isolates from Chinese chestnut trees were mostly moderately or highly pathogenic (Fig. 4). As found above (Fig. 3), most cankers were larger in August in these tests than in 1976 tests. Possibly, environmental conditions were more conducive to disease development in 1977 than in 1976. One isolate, ALA, that was obtained from an American chestnut tree in New York, appeared to be hypovirulent, but this cannot be stated conclusively as all trees inoculated with this isolate were killed by isolates obtained from Chinese chestnut trees, HF38 and HF20. The latter isolate appeared to be the most pathogenic of all isolates examined in this study, and suggests the possibility that this blight-resistant species may be colonized frequently by isolates of higher pathogenicity. The two isolates, HF38 and HF20, recovered from Chinese chestnut trees with severe cankers (Headland *et al.*, 1976), were highly pathogenic, while the third isolate examined, CCS, from a slightly diseased Chinese chestnut tree was moderately pathogenic (Fig. 2).

Our results suggest that most American chestnut trees examined in this study are not infected with hypovirulent *E. parasitica,* and thus, they may possess some degree of blight resistance. Studies are in progress to confirm this by more extensive pathogenicity trials and chemical and histopathological approaches, and to determine the possible role of certain environmental factors in the expression of blight resistance by American chestnut trees. American hypovirulent strains of *E. parasitica,* such as those from the W American chestnut tree in Virginia (Griffin *et al.*, 1977) and an Amer-

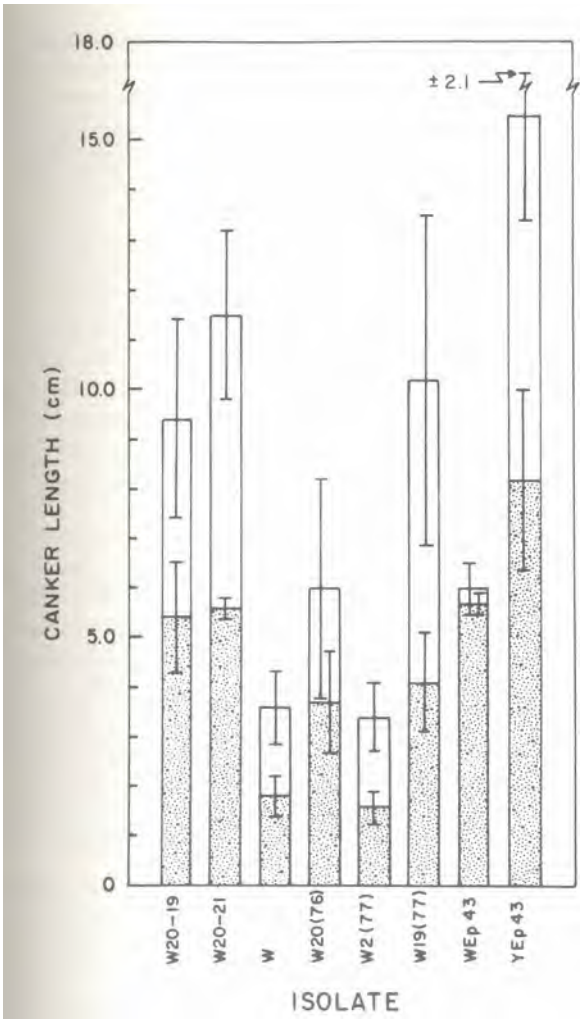

Figure 3. Canker lengths after 2.5 months (stippled bar) and 4.5 months (total bar) produced by W and Ep43 *Endothia parasitica* isolates on stump sprouts of American chestnut in 1977. Variation is indicated as standard error.

ican chestnut tree in Michigan (Elliston *et al., 1977),* may offer promise for control of blight on American chestnut. We suggest that these and other hypovirulent *E. parasitica* isolates, derived from European strains (Day *et al.,* 1977; Van Alfen *et al.,* 1975), may be more effective in controlling disease on blight-resistant American chestnut trees than on blight-susceptible American chestnut trees.

ACKNOWLEDGMENTS

Appreciation is expressed to Bruce Given, West Virginia Department of Agriculture; Al Dietz, Wadsworth, Ohio; Henry Plummer, Hampden, Maine; and Harry Hotine, Bayville, New York, for assistance in locating surviving American chestnut trees, and to Richard Jaynes for supplying the culture of Ep43. This research was supported in part by a grant from the Michaux Fund of the American Philosophical Society, Philadelphia. We thank Lucille Griffin for technical assistance and for translation of European journal articles.

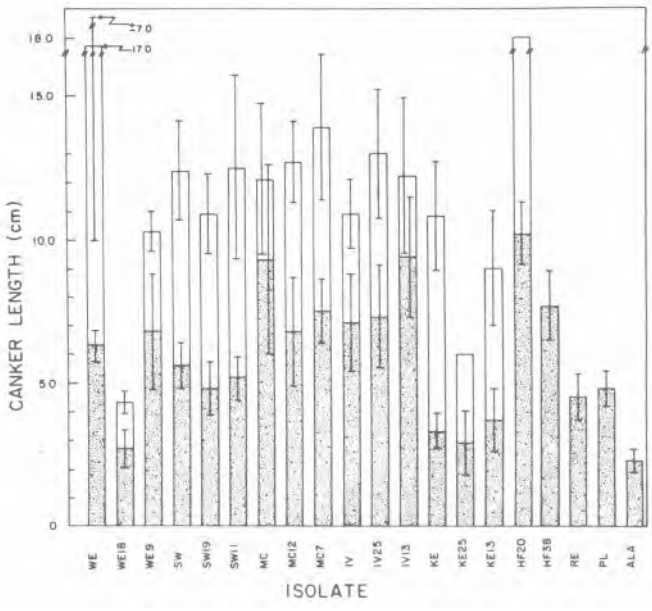

Figure 4. Canker lengths after 2.5 months (stippled bar) and 4.5 months (total bar) produced by *Endothia parasitica* isolates on stump sprouts of American chestnut in 1977. Variation is indicated as standard error.

LITERATURE CITED

Barnett, H. L. and V. G. Lilly.
1952. THE EFFECT OF COLOR OF LIGHT ON SPORULATION OF CERTAIN FUNGI. Proc. West Va. Acad. Sci. 24: 60-64.

Bonifacio, A. and T. Turchetti.
1973. DIFFERENYL MORFOLOGICE E FISIOLOGICHE IN ISOLATEI DI ENDOTHIA PARASITICA (MURR.). And. Ann. Acad. Ital. Sci. For. 22:111-131.

Day, P. R., J. A. Dodds, J. E. Elliston, R. A. Jaynes, and S. L. Anagnostakis.
1977. DOUBLE-STRANDED RNA IN ENDOTHIA PARASITICA. Phytopathology 67:1393-1396.

Elkins, J. R., J. H. Wright, and G. J. Griffin.
1977. RAPID SPECTROPHOTOMETRIC ANALYSIS OF TANNINS BASED ON THEIR REMOVAL FROM SOLUTION BY PRECIPITATING AGENTS. Proc. West Va. Acad. Sci. 49:9 (Abstr.).

Elliston, J. E., R. A. Jaynes, P. R. Day, and S. L. Anagnostakis.
1977. A NATIVE AMERICAN HYPOVIRULENT STRAIN OF ENDOTHIA PARASITICA. Proc. Amer. Phytopathol. Soc. 4:111 (Abstr.).

Grente, J. and S. Sauret.
1969. L'HYPOVIRULENCE EXCLUSIVE EST-ELLE CONTROLLEE PAR DIS DETERMINANTS CYTOPLASMIQUES? C. R. Acad. Sci. Paris 268:3173-3176.

Griffin, G. J., J. R. Elkins, G. S. Tomimatsu, and F. V. Hebard.
1977. VARIATION IN PATHOGENICITY OF AMERICAN ISOLATES OF ENDOTHIA PARASITICA ON AMERICAN CHESTNUT. Proc. Amer. Phytopath. Soc. 4:108 (Abstr.).

Headland, J. K., G. J. Griffin, R. J. Stipes, and J. R. Elkins.
1976. SEVERITY OF NATURAL ENDOTHIA PARASITICA INFECTION OF CHINESE CHESTNUT. Plant Dis. Rep. 60: 426-429.

Johansen, D. A.
1940. PLANT MICROTECHNIQUE. McGraw-Hill, New York, 523 pp.

Roane, M. K. and R. J. Stipes.
1976. THE FUNGAL GENUS ENDOTHIA IN THE SOUTHERN APPALACHIANS. Pages 227-242. *In* B. C. Parker and M. K. Roane, eds. The distributional history of the biota of the southern Appalachians. Part IV. Algae and fungi. Univ. Press of Virginia, Charlottesville, 416 pp.

Shear, C. L., N. E. Stevens, and R. J. Tiller.
1917. ENDOTHIA PARASITICA AND RELATED SPECIES. USDA Bull. 380. 82 pp.

Van Alfen, N. K., R. A. Jaynes, S. L. Anagnostakis, and P. R. Day.
1975. CHESTNUT BLIGHT: BIOLOGICAL CONTROL BY TRANSMISSIBLE HYPOVIRULENCE IN ENDOTHIA PARASITICA. Science 189:890-891.