Hypovirulent Isolates of *Endothia parasitica* Associated with Large American Chestnut Trees

R. A. JAYNES, Horticulturist, and J. E. ELLISTON, Assistant Plant Pathologist, Connecticut Agricultural Experiment Station, New Haven 06504

ABSTRACT

Jaynes, R. A., and Elliston, J. E. 1982. Hypovirulent isolates of *Endothia parasitica* associated with large American chestnut trees. Plant Disease 66:769-772.

Hypovirulent isolates of *E. parasitica* were associated with large, chronically infected, American chestnut trees. Normal isolates from such cankers were also commonly obtained. The abnormal isolates varied widely in pathogenicity. Although not all of the hypovirulent isolates had abnormal cultural characteristics, most showed considerable differences in pigmentation, growth rate, mycelial organization, and patterns of segregation of single-conidial isolates compared with normal. Several isolates did not produce perithecia in the field. Segregation of colony types among single-conidial isolates and presence of double-stranded RNA among several isolates suggested that viruslike cytoplasmic agents were responsible for their hypovirulence. The practical value that these isolates might have in the biological control of chestnut blight is still to be determined.

Additional key words: *Castanea dentata*, chestnut blight, cytoplasmic hypovirulence

American chestnut trees (*Castanea dentata* (Marsh.) Borkh.) resistant to the chestnut blight fungus (*Endothia parasitica* (Murr.) P.J. & H.W. Anderson) have been sought ever since the disease devastated the native population in the first half of this century. Periodic notes of optimism on the "return" of the chestnut have occurred for more than 50 yr (16,17). Scattered large American chestnut trees (20–100 cm diameter at breast height [DBH]) persist in the presence of the chestnut blight.

The chestnut blight fungus infects wounds and normally causes girdling cankers that kill susceptible trees. Small stems may be killed in one season, but trunks of large trees may not be killed for 3–5 yr. Sprouts commonly are produced below infections and from the root collar of infected trees. These usually become infected before they attain a DBH of 15 cm, although blightfree or recently infected stems as large as 30 cm DBH are occasionally found.

Large, blighted trees (>20 cm DBH) that persist for years have been an anomaly. Several investigators suggested that such trees might be genetically resistant (10,11,14), but this has not yet been demonstrated.

In Europe, gradual remission of the chestnut blight disease on the susceptible European chestnut (*C. sativa* Mill.) has been associated with hypovirulent isolates of the fungus that contain viruslike, cytoplasmic factors (4,12,13); thus the name cytoplasmic hypovirulence (CH) (8). The situation in Europe and research on these isolates have been reviewed recently (1,3,8).

Hypovirulent isolates were first obtained in 1976 from large, surviving American trees in Rockford, MI, outside the natural range of *C. dentata* (9). Since then, others have been reported from locations within the native range (14). Dodds (6) and Elliston and Dodds (*unpublished*) analyzed American CH isolates from Rockford and from Cumberland Mountain, TN, and concluded that the agents affecting the American CH isolates were different from those in European isolates. Dodds (5) published photographs of a possible viruslike particle from one European hypovirulent isolate.

We present evidence suggesting that hypovirulent isolates of *E. parasitica* may be partly responsible for the survival of large, chronically infected American chestnut trees in the eastern United States.

Accepted for publication 4 December 1981.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

0191-2917/82/090769O4/$03.00/0

MATERIALS AND METHODS

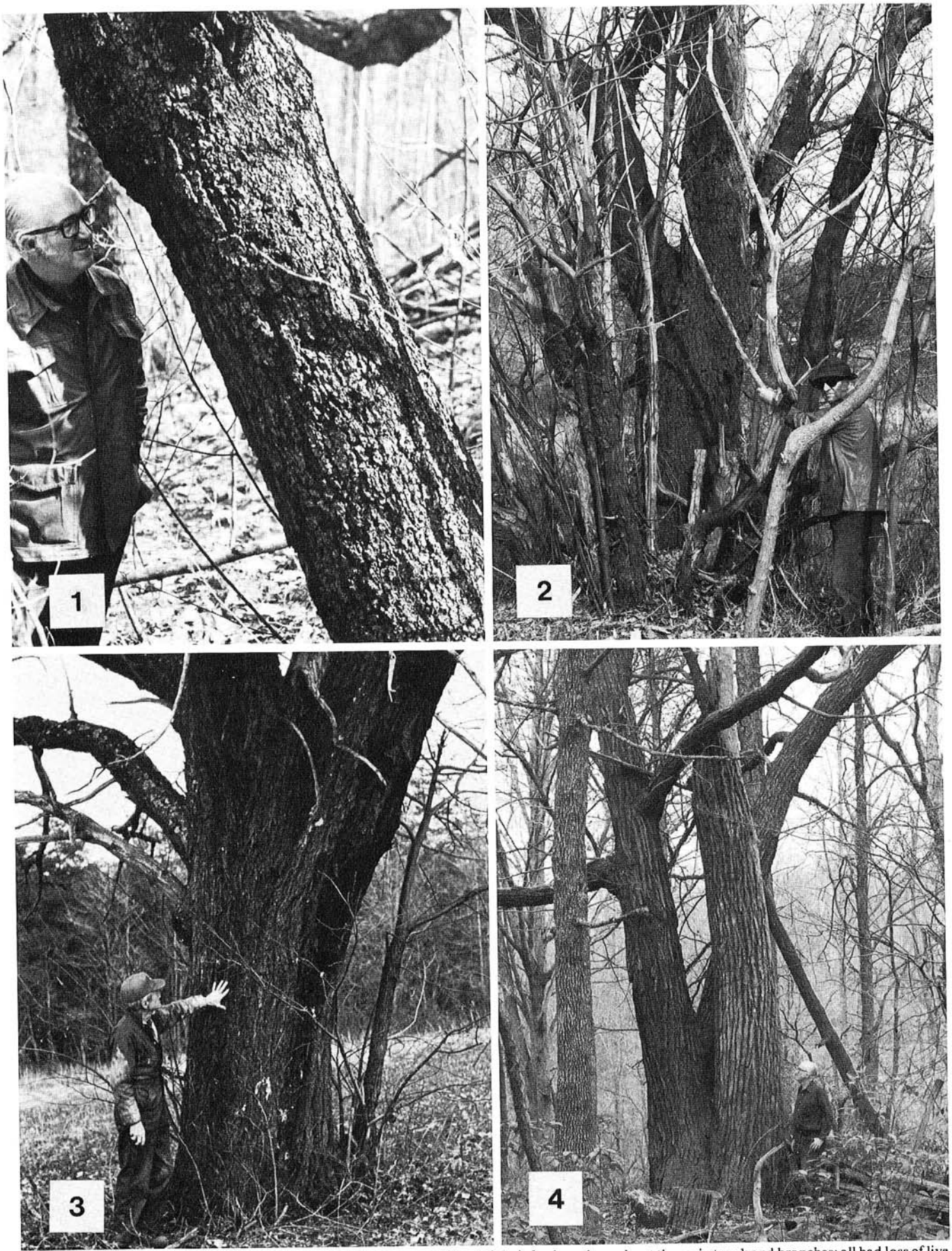
Trees and symptoms. American chestnut trees from sites in seven states were studied. Stem diameter of most trees (13–90 cm DBH) was unusually large, and source cankers were generally persistent, heavily callused, and in many cases diffuse and superficial, covering much of the surface of the main trunk and primary branches. Fruiting structures, pycnidia and perithecia, were not abundant compared with normal cankers. The trees were often disfigured with cankers, dead branches, and girdled sprouts. Most trees were confirmed as American chestnuts by examining twig samples. Others were identified by cooperating scientists and foresters.

Bark samples and isolations. We obtained bark samples measuring 3–15 cm^2 and containing live and infected tissue from one or more cankers and from one or more trees per site. Isolates resembling *E. parasitica* were obtained by slicing off the surface layer of bark and transferring 1–2 mm^3 of infected tissue to petri plates of potato-dextrose agar supplemented with biotin and methionine (PDAbm) (18). One to several isolates were obtained per canker, and one or more were studied. Cultures were selected if they had abnormal pigmentation, sporulation, or growth rate.

Pathogenicity. All isolates except Ep700 and 701 were tested several times between 1976 and 1981 for pathogenicity on American chestnut stems. Excised stems were used for short-term tests (4–6 wk) during the dormant season (7). Other tests up to 16 wk in duration during the growing season were with sprouts measuring 4–10 cm diameter in the field. Inoculations were as follows: an 8-mm-diameter disk of bark to the depth of the cambium was removed with a cork borer; the hole was filled with agar containing mycelium and covered with masking tape (15). Inoculations with each strain in each test were replicated three or four times. In each test, several putative CH isolates were compared with one or more normal isolates. Canker areas were determined from length and width measurements using the formula for an ellipse.

Perithecia and ascospores. The isolates were inoculated (three replicates) into American chestnut sprouts in the field in June, and infected bark was sampled in December for determination of perithecium and ascospore formation. Length and width of 25 spores from each of three cankers per isolate were measured.

Culture morphology. Morphologies of all cultures were compared several times with that of one or more normal isolates under standard light and temperature conditions, 20 C under a 16-hr photo-



Figs. 1–4. Representative American chestnut trees with persistent, diffuse blight infections throughout the main trunk and branches; all had loss of live crown: **(1)** Ragged Mountain, VA (Ep170); a dead stem of about 30 cm diameter at breast height and live basal sprouts were also present. **(2)** Fairfax, VA (Ep209, 213); many dead and live stems present. **(3)** Amherst, VA; large sprout on right also had an extended, callused canker. Ep172 was collected from a canker on a nearby tree. **(4)** Madison, VA (Ep198); bark is infected but not as disfigured as in 2 and 3.

period for 5–9 days after transfer to PDAbm.

Presence of dsRNA. All isolates listed in Table 1 were analyzed for double-stranded (ds) RNA following Dodd's technique (6) as modified by N. DePalma (*personal communication*). Electrophoresis was at 4 mA/hr for 10 hr on 5% polyacrylamide gels Gels were stained with propidium iodide and examined under ultraviolet light. Strains known to contain dsRNA and lacking dsRNA were included with test isolates in each set analyzed.

Single spore isolation. To assist in determining whether the culture abnormalities observed were under nuclear or cytoplasmic control, single-spore (conidium) isolates were obtained and the culture types classified. Cultures for single-conidial isolation experiments were grown on PDAbm for 2 wk at 20 C. Conidia were harvested in sterile deionized water, and suspensions were filtered through sterile cheesecloth and diluted serially. One-milliliter aliquots of dilutions containing approximately 15–120 spores per milliliter were plated on complete medium (18). Three plates were seeded with each concentration and incubated 48 hr at 24–26 C. Isolations were made from plates containing 30–100 well-separated germlings to yield a total of 50–120 isolates in most experiments. Germlings were located with a dissecting microscope at ×60 magnification, and all single germlings were removed from each plate to ensure randomness. Isolated germlings were incubated individually on plates of PDAbm at 20 C, and colonies were scored for morphological type after 7–9 days. Colonies of appropriate normal and dsRNA-containing strains were grown with the single-spore isolates to provide standards for comparison.

RESULTS

Table 1 summarizes information on the trees and some of the fungal isolates. Representative trees and cankers are illustrated in Figures 1–4.

Pathogenicity. Results of nine pathogenicity tests are presented in Table 1. Average pathogenicity of individual isolates over all tests ranged from 3 to 73% of the normal strain. Variation, especially from test to test, was great. Differences in duration of the test, time of year, and differences among host stems and location on a stem account for some of the variation. But some variation also reflects the instability of some isolates as explained below.

Culture morphology. Elliston (7) described the morphology of normal and several abnormal isolates. Most but not all the isolates studied (Table 1) were abnormal. A few were slow-growing, pigmented, and had abnormal arrangements of pycnidia (such as Ep 60), whereas others lacked pigment or showed some other irregularity, such as weak growth or sectoring. A few cultures (such as Ep 198, 213, and 255) may have lost their hypovirulence as evidenced by changes in culture morphology or pathogenicity. Others (such as 223) may have sectored or changed to a more virulent but not fully normal state. Although duplicate sets of stock cultures of each strain were established shortly after isolation, instability of some strains was such that, after 2–4 yr, characteristics of the original isolate were lost.

Perithecia and ascospores. Several isolates did not produce perithecia (Table 1). Failure to produce perithecia is characteristic of European and many American hypovirulent isolates (7,21). The perithecia produced had characteristics typical of *E. parasitica* (19). Some consistent differences in ascospore size appeared to be associated with specific isolates (Table 1). For example, Ep 172 had large ascospores compared with those of Ep 223, yet the variation falls within that previously reported for *E. parasitica* (2,19).

Presence of dsRNA. Double-stranded RNA was detected in 14 of the 22 hypovirulent isolates tested (Table 1). Patterns of dsRNA on gels differed with the isolate but were representative of the types previously reported (6).

Single spore isolation. Cytoplasmically controlled abnormalities often segregate among asexual progeny of haploid homokaryotic strains, yielding mixtures

Table 1. Characteristics of selected isolates resembling *Endothia parasitica* from large, surviving American chestnut trees

Culture collection number	Source	DBH[a] of source tree (cm)	Pathogenicity when inoculated on American chestnut stems (canker area)		Culture morphology[b]	Ascospore length × width[c] (μm)	dsRNA detected	No. of single-conidial colony types
			% Normal	No. of tests				
29[d]	Guilford, CT	8	100	9	N	9.0 × 4.1	−	1
60	Rockford MI	15	3	5	Abn	None	+	3
88	Rockford MI	26	67	3	Abn	None	+	NT[e]
170	Ragged Mountain, VA	59	13	6	Abn	None	+	3
171	Holland, MI	25	13	7	Abn	None	+	5
172	Amherst County, VA	13[f]	68	4	~N	9.1 × 4.3	+	1
173	Grand Haven, MI	90	70	5	~N	None	+	1
174	Newtown Square, PA	30	52	5	~N	8.5 × 4.0	+	1
198	Madison, VA	76	66	6	Abn	8.3 × 3.9	+	1[g]
204	Plum Point, MD	60	16	4	Abn	8.4 × 4.0	+	2
206	Rochester, NY	37	62	5	~N	8.1 × 4.0	+	1
209	Fairfax, VA	61	34	5	N	8.4 × 4.1	+	NT
213	Fairfax, VA	61	73	6	Abn	8.8 × 4.1	−	Many
216	New Fairfield, CT	34	39	5	Abn	9.1 × 4.1	−	NT
217	New Fairfield, CT	34	10	4	Abn	NT	−	NT
221	Roxbury, CT	20	46	5	~N	8.7 × 4.0	−	NT
222	Roxbury, CT	20	27	5	~N	8.5 × 3.9	−	NT
223	Roxbury, CT	20	44	5	Abn	8.4 × 3.6	−	NT
234	Cumberland, TN	73	4	4	Abn	None	+	2
252	Cumberland County, PA	17	52	4	Abn, white	8.4 × 3.9	−	NT
254	Rochester, NY	30	14	3	Abn	None	+	NT
255	Rochester, NY	16	42	4	~N	None	−	NT
700	Natural Bridge, VA	18	3	1	Abn	NT	+	2
701	Natural Bridge, VA	18	NT	NT	~N	NT	+	NT

[a] Diameter at breast height.
[b] See Elliston (7) for more detailed description of normal (N) and abnormal (Abn) culture appearance. ~N = near normal.
[c] Mean of 25 spores. Standard errors were ± 0.08–0.19 for length and ± 0.03–0.09 for width.
[d] Other normal strains with characteristics similar to 29 were also used for comparative purposes.
[e] Not tested.
[f] Adjacent to a 96-cm (DBH) American chestnut (Fig. 3).
[g] Abnormal, like isolate 198.

of normal and abnormal isolates (13), whereas those traits under nuclear control will not segregate. Of thirteen isolates examined (last column, Table 1), most of the morphologically abnormal isolates segregated two or more kinds of colonies, ie, normal and one or more abnormal types. Exceptions were Ep 174, an abnormal type that produced all normal type isolates, and Ep 198, which consistently produced only Ep 198-like abnormal colonies. Ep 174 may represent a CH type in which the abnormal cytoplasmic factor is excluded from most conidia. Ep 198 may be a nuclear variant and could even be another *Endothia* species.

DISCUSSION

The host-parasite system of *C. dentata* and *E. parasitica* has been highly imbalanced since the fungus was introduced into the United States in the late 1800s. Even if the American chestnut is eliminated, *E. parasitica* would likely remain, surviving as a saprophyte or weak parasite on other hosts, including native oaks and introduced, blight-resistant chestnut species.

Changes in host, parasite, or both towards a more "balanced" relationship would have to occur to permit the American chestnut population to increase substantially and for the tree to regain its usefulness. The situation in Italy suggests that changes in the fungus have brought about remission (not elimination) of the disease there (20). The existence of large American chestnut trees surviving for many years with persistent *E. parasitica* infections suggests that such trees may have higher than normal genetic resistance, that the strains of the fungus infecting them may have reduced virulence, or both; ie, the necessary changes may be occurring.

The results of this study do not permit a clear choice among the possible explanations for survival of large trees, but the results indicate that many of the isolates from these trees are abnormal. Most of the isolates are less pathogenic than representative normal strains, and many of them contain dsRNA. Isolates with reduced virulence and without detectable dsRNA could be genetically deficient (ie, nuclear hypovirulent strains) or they may contain very low levels of dsRNA that were not detected in these tests. Further tests are required to determine whether the dsRNA or defective nuclear genome in the fungus, or both, are responsible for an isolate's reduced virulence. The levels of resistance of the trees are also unknown but could be tested using vegetatively propagated plants.

The trees from which hypovirulent isolates were obtained are not thrifty, healthy, and well formed. Yet the presence of hypovirulent strains apparently prevents normal strains from killing the trees. Whether hypovirulent strains associated with surviving American chestnut trees can be used effectively to grow large, useful American chestnut trees remains to be demonstrated.

Persistent cankers are a likely source of inoculum for spreading and establishing CH strains. These cankers often contain a mixture of hypovirulent and normal *E. parasitica* strains, as indicated by the range of isolate types obtained. When a single hypovirulent strain is isolated, cultured, and then reinoculated into a tree, usually the kind of canker from which the strain was isolated is not produced (7; *unpublished*). The reasons for this have not been determined. As a means to establish persistent hypovirulent cankers, G. Kuhlman (*personal communication*) suggested grafting intact pieces of bark from such cankers onto susceptible trees. The integrity of the host-strain complex would be maintained and transferred to the new host tree, and the resulting persistent cankers could serve as sources of hypovirulent inocula in areas where only normal strains now exist. This hypothesis is being tested.

Our results suggest that strains containing dsRNA are more common in the middle and southern parts of the natural range of chestnut and outside its natural range than in New England. However, since the causes of hypovirulence may be many, this observation may be irrelevant. Thorough testing of many isolates from each large surviving tree is necessary to make valid conclusions as to the occurrence and significance of hypovirulent strains. It is likely a false hope to expect to categorize them all with a single set of parameters (7).

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of Nancy DePalma and Barbara Wooding. We thank Gordon Allen, Larry Brewer, Dick Cook, Tom Dierauf, George Kuhlman, David McCarroll, Barry Towers, and others who have directed us to surviving trees or have supplied information and samples from them.

LITERATURE CITED

1. Anagnostakis, S. 1978. The American chestnut: New hope for a fallen giant. Conn. Agric. Exp. Stn., New Haven, Bull. 777. 9 pp.
2. Anderson, P. J. 1914. The morphology and life history of the chestnut blight fungus. Pa. Chestnut Tree Blight Comm. Bull. 7. 44 pp.
3. Day, P. R., and Dodds, J. A. 1979. Viruses of plant pathogenic fungi. Pages 201-238 in: Viruses and Plasmids in Fungi. P. A. Lemke, ed. M. Dekker, New York. 653 pp.
4. Day, P. R., Dodds, J. A., Elliston, J. E., Jaynes, R. A., and Anagnostakis, S. L. 1977. Double-stranded RNA in *Endothia parasitica*. Phytopathology 67:1393-1396.
5. Dodds, J. A. 1980. Association of type I viral-like dsRNA with club-shaped particles in hypovirulent strains of *Endothia parasitica*. Virology 107:1-12.
6. Dodds, J. A. 1980. Revised estimates of the molecular weights of dsRNA segments in hypovirulent strains of *Endothia parasitica*. Phytopathology 70:1217-1220.
7. Elliston, J. E. 1978. Pathogenicity and sporulation of normal and diseased strains of *Endothia parasitica* in American chestnut. Pages 95-100 in: Proc. Am. Chestnut Symp. West Virginia University, Morgantown.
8. Elliston, J. E. 1981. Hypovirulence and chestnut blight research: Fighting disease with disease. J. For. 79:657-660.
9. Elliston, J. E., Jaynes, R. A., Day, P. R., and Anagnostakis, S. L. 1977. A native American hypovirulent strain of *Endothia parasitica*. (Abstr.) Proc. Am. Phytopathol. Soc. 4:111.
10. Given, J. B., and Haynes, S. C. 1978. The West Virginia Dept. of Agriculture American chestnut program. Pages 41-42 in: Proc. Am. Chestnut Symp. West Virginia University, Morgantown.
11. Gordon, J., Allen, G., and Brown, P. 1974. The Port Republic group of American chestnut trees. North. Nut Grow. Assoc., Annu. Rep. 65:89-95.
12. Grente, J. 1975. La lutte biologique contre le chancre du chataignier par "hypovirulence contagieuse." Ann. Phytopathol. 7:216-218.
13. Grente, J., and Sauret, S. 1969. L'hypovirulence exclusive, phenomene original en pathologie vegetale. C. R. Hebd. Seances Acad. Sci. Ser. D. 268:2347-2350.
14. Griffin, G. J., Elkins, J. R., Tominatsu, G., and Hebard, F. 1978. Virulence of *Endothia parasitica* isolated from surviving American chestnut trees. Pages 55-59 in: Proc. Am. Chestnut Symp. West Virginia University, Morgantown.
15. Jaynes, R. A., and Elliston, J. A. 1980. Pathogenicity and canker control by mixtures of hypovirulent strains of *Endothia parasitica* in American chestnut. Phytopathology 70:453-456.
16. Kelly, A. P. 1924. Chestnut trees surviving blight. Science 60:292-293.
17. Kelly, A. P. 1944. The present status of American chestnut in southeastern Pennsylvania. Landenberg Laboratory, Landenberg, PA. 11 pp.
18. Puhalla, J. E., and Anagnostakis, S. L. 1971. Genetics and nutritional requirements of *Endothia parasitica*. Phytopathology 61:169-173.
19. Shear, C. L., Stevens, N. S., and Tiller, R. J. 1917. *Endothia parasitica* and related species. U.S. Dep. Agric. Bull. 380. 82 pp.
20. Turchetti, T. 1978. Some observations on the "hypovirulence" of chestnut blight in Italy. Pages 92-94 in: Proc. Am. Chestnut Symp. West Virginia University, Morgantown.
21. Willey, R. L., and MacDonald, W. L. 1980. Growth and sporulation of virulent and hypovirulent isolates of *Endothia*. (Abstr.) Phytopathology 70:694.