A Rapid Method for Testing the Virulence of *Cryphonectria parasitica* Using Excised Bark and Wood of American Chestnut

J. K. Lee, T. A. Tattar, P. M. Berman, and M. S. Mount

Graduate research assistant, professor, research associate, and professor, respectively, Department of Plant Pathology, University of Massachusetts, Amherst 01003.

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ABSTRACT

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A rapid and reproducible method for testing the virulence of *Cryphonectria parasitica* using American chestnut (*Castanea dentata*) was developed. Bark- and wood-tissue samples excised from American chestnut tree stems were inoculated with virulent and hypovirulent *C. parasitica* strains. Samples were incubated at 25 C for 4 days, and virulence was assessed as the area of bark and wood tissue having brown, necrotic cells. Hypovirulent strains damaged an area 2.16 cm^2 or less of each tissue sample, while virulent strains consistently damaged a greater area (3.62–3.67 cm^2). Living trees and excised stems were inoculated with the same fungal strains; the test results were compared to those from bark/wood tests. The virulence-assessment data from trees and cut stems correlated well with bark/wood data. However, the bark/wood-tissue test was superior in terms of the incubation time required, convenience, and data reproducibility. This procedure should be extremely useful as an initial screening of *C. parasitica* strains for hypovirulence.

Additional keywords: biological control, chestnut blight.

The Chestnut blight fungus, *Cryphonectria parasitica* (Murrill) Barr, is responsible for a lethal disease affecting American chestnut trees (*Castanea dentata* (Marsh.) Borkh.). *C. parasitica* strains that cause only limited damage to trees, hypovirulent (H) strains, have been isolated (2,10). Active chestnut blight cankers have been arrested by direct treatments with H strains (14,15). The H strains contain dsRNA viruses transferrable to virulent (V) strains by hyphal anastomosis (1,11,13). Acquisition of these viruses can convert V strains to hypovirulent. To study and evaluate *C. parasitica* hypovirulence, a rapid, convenient, and reproducible virulence test is critical. Standard tests involve inoculation of the fungus into living trees (3,7), excised stems (4–6), or apple fruit (4,5), with incubation times of 2–3 mo, 5 wk, and 3 wk, respectively. Field research on living trees can be difficult because several healthy trees of similar size and developmental stage must be found for replicate inoculations with the fungus. Genetic variation among the trees can introduce another variable to this test. In addition, canker development from field inoculations can be poor during late fall and winter. The cut-stem method, in which comparable cut-stem sections from a tree (or trees) are inoculated with fungi in the laboratory, can be used to overcome the seasonal limitations of field tests and to minimize size, developmental-stage, and genetic variations. However, the cut-stem virulence-test incubation period is also long and requires constant, high humidity. Results from apple-fruit inoculation tests can be obtained more quickly, but because this tissue is substantially different from chestnut, the conclusions drawn from the fruit data may not always be directly applicable to chestnut. McCarroll and Thor (12) found that inner-bark tissue from American chestnut has a detectable reaction to fungal products in vitro. As a result, this type of tissue has potential for use in fungal virulence tests. In our study, we used excised bark- and wood-tissue sections from American chestnut trees to develop an efficient *C. parasitica* virulence test and then compared the results with data from inoculations into living trees and cut-stem sections. Some of the material in this paper has been presented in abstracts (8,9).

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MATERIALS AND METHODS

Fungal isolates and cultural conditions. Virulent strains were isolated from bark samples of diseased American chestnut trees in Pelham (PE) and Shutesbury (SB), MA (8,9). Four V strains, each from a different vegetative-compatibility group, were used in this study: PE20, PE23, PE51, and SB2. Hypovirulent strains AA3, CP802, and CP748 were provided by S. L. Anagnostakis (Connecticut Agricultural Experiment Station, New Haven). The H strains harbor dsRNA viruses of European origin (S. L. Anagnostakis, *personal communication*). Hypovirulent strain SB2(748) was produced by pairing V strain SB2 with H strain CP748 on agar (8,9). Fungal strains were grown on Difco potato-dextrose agar (PDA) in petri dishes for 5 days at 25 C. Stock cultures were maintained on PDA slants at 4 C. A sterile cork borer was used to cut 5-mm-diameter plugs of agar with mycelium from the advancing edge of actively growing colonies to provide inoculum for virulence tests.

Living-tree inoculation. In early April, American chestnut trees (2.3–4.3 cm dbh) were inoculated as described by Elliston (3). Fungal-mycelium agar plugs were placed into holes cut in the bark, and each site was covered with water-proof tape. A trial consisted of inoculating each fungal strain into four different trees at a selected location. Four trials, each at a different field location, were performed per strain. After 2 mo, the length and width of each canker was measured, and the elliptical canker area (square centimeter) was calculated.

Excised-stem inoculation. Inoculations of cut-stem sections were performed as described by Fulbright (5). In early March, stems 2–3 cm in diameter were harvested, cut into 30-cm-long pieces, and the cut ends sealed with Parafilm. Three bark plugs (12 cm distant from each other) were removed from each stem piece with a 5-mm cork borer, and agar plugs with mycelium were inserted. The inoculation sites were then sealed with Parafilm to prevent desiccation, and the samples were placed in the dark

at 25 C with 95% humidity. A trial consisted of inoculating each fungal strain into four stem pieces, each piece from one of four different trees at a selected field location. In total, four trials were performed using samples collected from four different field locations. After 5 wk, the length and width of the cankers were measured, and the elliptical canker areas (square centimeter) were calculated.

Bark- and wood-tissue sections. Excised stems were harvested from trees in early March and immediately placed upright, with the bottom end in water and the top end sealed with Parafilm. The sealed stems were stored at 4 C and used within 4 wk for bark/wood sampling. Stems were cut into 2.5-cm-long sections, the pieces were bisected longitudinally, and the bark and wood tissue were separated using forceps. An agar plug 5 mm in diameter was placed, fungal-mycelium side down, in the center of each inner-bark- and wood-tissue section. Samples were then incubated in the dark at 25 C on moistened filter papers inside petri dishes (Fig. 1). A trial consisted of inoculating each fungal strain into four bark pieces and four wood pieces (each bark/wood piece was collected from four different trees at a selected field location). A total of four trials, using material collected from different field locations, was carried out. After 4 days, the zone containing brown, necrotic cells was measured, and the elliptical necrotic area (square centimeter) was calculated. Samples were then incubated for an additional 2.5 wk to see if pycnidia would develop.

RESULTS

The size of cankers resulting from fungal inoculations on both living trees and excised stems varied widely among the fungal strains (Fig. 2). In general, however, the cankers produced by V strains on both trees and excised stems were larger than those produced by H strains. Also, 2 mo after inoculation of living trees with the fungus, cankers at the wound sites on V-strain infected trees appeared noticeably different from those on H-strain infected trees. Virulent cankers had orange pycnidia at the wound site, and the cankers were slightly sunken. The H strain CP802 was the only notable exception. Although CP802 has been confirmed as hypovirulent (S. L. Anagnostakis, *personal communication*), when cultured on PDA, CP802 grows considerably faster than the majority of H strains. In addition, the colony color of CP802 on PDA is yellow-orange (as are most V strains), while most of the H strains are closer to white. Also, the canker size produced by CP802 on both living trees and excised stems was larger than cankers produced by other H strains (Fig. 2). However, other than canker size, the characteristics of cankers caused by CP802 on both living trees and excised stems resembled those caused by the other H strains.

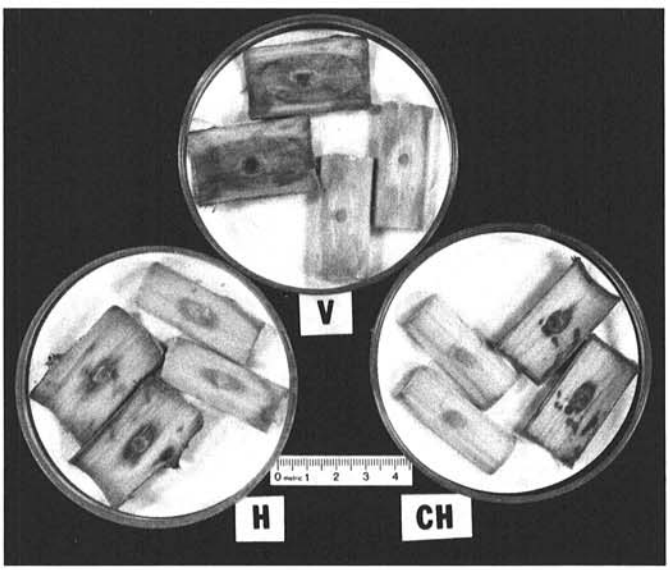

Fig. 1. Bark/wood-tissue virulence test of *Cryphonectria parasitica* strains on American chestnut (*Castanea dentata*). Agar plugs with fungal mycelium were placed on 2.5-cm-long excised bark- and wood-tissue sections. Samples were incubated in the dark at 25 C on moistened filter papers inside petri dishes. V is virulent strain PE23, and H is hypovirulent strain AA3. CH is hypovirulent strain PE23(AA3), which was produced by converting PE23 to hypovirulence via pairing with AA3. This photograph was taken 3 days after inoculation with the fungal strains.

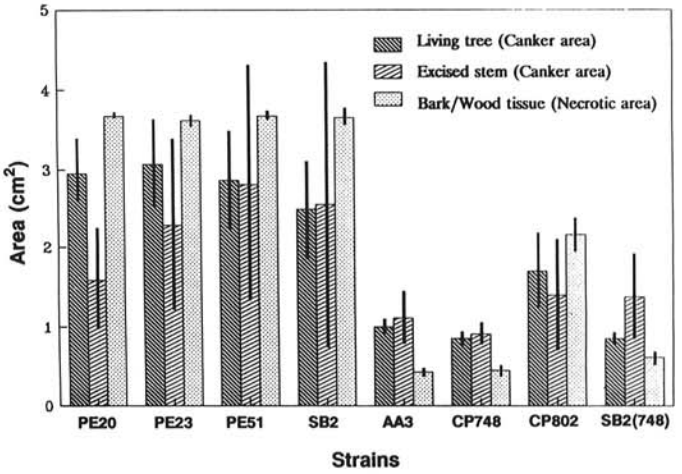

Fig. 2. Virulence testing of *Cryphonectria parasitica* on American chestnut (*Castanea dentata*). Agar plugs with fungal mycelium were used to inoculate living trees, excised-stem sections, and isolated 2.5-cm-long bark- and wood-tissue sections. Incubation times were 2 mo, 5 wk, and 4 days, respectively. The area (square centimeter) of cankers on living trees and excised stems and the area (square centimeter) of necrotic tissue on bark/wood samples were determined. The data presented here are the averages of replicates from four trials of each virulence test per strain. Standard deviation is illustrated by a vertical line on the top of each bar on the graph. *C. parasitica* strains PE20, PE23, PE51, and SB2 are Massachusetts virulent strains. Strains AA3, CP748, and CP802 are hypovirulent strains provided by S. L. Anagnostakis (Connecticut Agricultural Experiment Station, New Haven). Strain SB2(748) is a hypovirulent strain produced by pairing virulent strain SB2 with hypovirulent strain CP748.

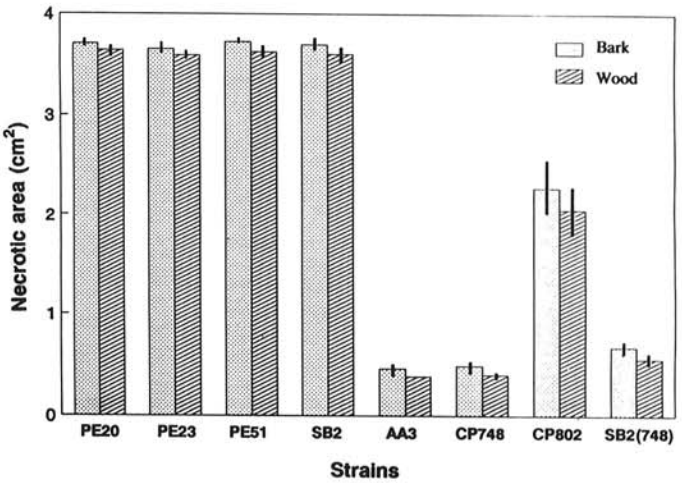

Fig. 3. Virulence tests of *Cryphonectria parasitica* on bark tissue and on wood tissue excised from American chestnut (*Castanea dentata*). Agar plugs with fungal mycelium were placed on 2.5-cm-long excised bark- and wood-tissue sections. Samples were incubated in the dark at 25 C on moistened filter papers inside petri dishes. After 4 days, the zone containing necrotic cells was measured and the necrotic area (square centimeter) was calculated. The data presented here are the averages of replicates from four trials per strain. Standard deviation is illustrated by a vertical line on the top of each bar on the graph. *C. parasitica* strains PE20, PE23, PE51, and SB2 are Massachusetts virulent strains. Strains AA3, CP748, and CP802 are hypovirulent strains provided by S. L. Anagnostakis (Connecticut Agricultural Experiment Station, New Haven). Strain SB2(748) is a hypovirulent strain produced by pairing virulent strain SB2 with hypovirulent strain CP748.

The bark- and wood-tissue tests gave clearer and more consistent results than the living-tree and excised-stem section tests (Fig. 2). On both bark and wood samples (Fig. 3), the necrotic area caused by H-strain infections never exceeded 2.1 cm^2, while V-strain necrotic areas were consistently larger (>3.6 cm^2). The data from inoculations of each *C. parasitica* strain on excised-bark tissue were consistently similar to the data from inoculations with the same fungal strain on excised-wood tissue (Fig. 3). The correlation coefficient of the bark- and wood-data sets from Figure 3 is 0.997 (Pearson correlation matrix). Therefore, the bark- and wood-tissue data for each fungal strain were combined for comparison with the living-tree and excised-stem data for that strain (Fig. 2). The correlation coefficient of the bark/wood data was 0.909 with the cut-stem data and 0.981 with the living-tree data. The correlation coefficient of the living-tree data with the cut-stem data was 0.879. As a result, although all the data sets correlate well, the bark/wood test and living-tree test had the closest correlation. Comparisons between single trials of each of the three virulence tests using several other V and H strains of *C. parasitica* (data not included) support these results.

On both bark and wood, V strains always produced abundant, spreading mycelium that covered the tissue (Fig. 1). When the incubation time for the bark/wood tests was extended to 3 wk, pycnidial development followed. The H strains, in general, did not produce abundant mycelium or any pycnidia, even after 3 wk of incubation on the bark/wood tissue. The only exception was the faster-growing, yellow-orange CP802, which did show minimal pycnidial development after extended incubation. However, a white H strain that grew even faster on PDA than CP802 produced a much smaller necrotic area than CP802 on bark/wood tissue and produced no pycnidia after extended incubation (data not included).

Finally, no significant differences were found in data from the bark and wood virulence tests among the trees collected at the four field locations (F distribution test, $P = 0.05$).

DISCUSSION

The bark/wood-tissue test offers many advantages over other *C. parasitica* virulence tests. Within only 4 days of inoculation with the fungi, an accurate estimation of virulence can be made. The results are clear, consistent, and correlate well with results from other virulence-test methods using the same fungal isolates. Colony morphology, growth characteristics, canker size produced, etc. can vary widely among H strains, possibly due to differences in their infecting viruses (10). Results from the bark/wood test more clearly predicted the hypovirulence of the atypical H strain, CP802, which contains dsRNA. In addition, several fungal strains, replicates per strain, and control strains previously identified as either V or H, can be tested on tissue collected from a single tree by using the bark/wood test. This is economical, saves time, and can eliminate any possible genetic variability among tissue samples. Alternatively, the bark/wood virulence-test samples need not be confined to a single tree for accuracy. Our study did not detect variation among bark/wood-tissue samples collected from different trees.

The degree of hypovirulence exhibited by different strains of *C. parasitica* varies widely; true hypovirulence can only be determined conclusively by numerous inoculations and long incubations of the fungus on living trees in the field. Also, the presence of the virus so often associated with hypovirulence can only be confirmed decisively by isolating nucleic acids from the infected fungi. However, the bark/wood-tissue test should prove to be extremely useful as an initial screening method to quickly select strains of *C. parasitica* with good hypovirulence potential.

LITERATURE CITED

1. Anagnostakis, S. L. 1984. The mycelial biology of *Endothia parasitica*. I. Nuclear and cytoplasmic genes that determine morphology and virulence. Pages 353-366 in: The Ecology and Physiology of the Fungal Mycelium. D. H. Jennings and A. D. M. Rayner, eds. Cambridge University Press, Cambridge.
2. 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.
3. 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. W. L. MacDonald, F. C. Cech, J. Luchok, and C. Smith, eds. West Virginia University Books, Morgantown.
4. Elliston, J. E. 1985. Characteristics of dsRNA-free and dsRNA-containing strains of *Endothia parasitica* in relation to hypovirulence. Phytopathology 75:151-158.
5. Fulbright, D. W. 1984. Effect of eliminating dsRNA in hypovirulent *Endothia parasitica*. Phytopathology 74:722-724.
6. Fulbright, D. W., Weidlich, W. H., Haufler, K. Z., Thomas, C. S., and Paul, C. P. 1983. Chestnut blight and recovering American chestnut trees in Michigan. Can. J. Bot. 61:3164-3171.
7. Griffin, G. J., Elkins, J. R., Tomimatsu, G., and Hevard, F. V. 1978. Virulence of *Endothia parasitica* isolated from surviving American chestnut trees. Pages 55-60 in: Proc. Am. Chestnut Symp. W. L. MacDonald, F. C. Cech, J. Luchok, and C. Smith, eds. West Virginia University Books, Morgantown.
8. Lee, J. K., Tattar, T. A., Berman, P. M., and Mount, M. S. 1991. Comparison of *Cryphonectria parasitica* strains from Massachusetts in cultural characteristics, pathogenicity, and phenol oxidase activity. (Abstr.) Phytopathology 81:1206-1207.
9. Lee, J. K., Tattar, T. A., Berman, P. M., and Mount, M. S. 1991. Vegetative compatibility and hypovirulence conversion of *Cryphonectria parasitica* on American chestnut trees in Massachusetts. (Abstr.) Phytopathology 81:1206.
10. MacDonald, W. L., and Fulbright, D. W. 1991. Biological control of chestnut blight: Use and limitations of transmissible hypovirulence. Plant Dis. 57:656-661.
11. Martin, R. M., and Van Alfen, N. K. 1991. The movement of viral-like RNA between colonies of *Cryphonectria parasitica*. Mol. Plant-Microbe Interact. 4:507-511.
12. McCarroll, D. R., and Thor, E. 1985. Do "toxins" affect pathogenesis by *Endothia parasitica*? Physiol. Plant Pathol. 26:357-366.
13. Newhouse, J. R., and MacDonald, W. L. 1991. The ultrastructure of hyphal anastomoses between vegetatively compatible and incompatible virulent and hypovirulent strains of *Cryphonectria parasitica*. Can. J. Bot. 69: 602-614.
14. Scibilia, K. L., and Shain, L. 1989. Protection of American chestnut with hypovirulent conidia of *Cryphonectria (Endothia) parasitica*. Plant Dis. 73:840-843.
15. Van Alfen, N. K. 1988. Viruses of *Endothia parasitica*. Pages 371-385 in: Viruses of Fungi and Simple Eukaryotes. Y. Koltin and M. J. Leibowitz, eds. Marcel Dekker, Inc., New York.