Protection of American Chestnut with Hypovirulent Conidia of *Cryphonectria (Endothia) parasitica*

K. L. SCIBILIA, Former Graduate Research Assistant, Department of Forestry, and L. SHAIN, Professor, Department of Plant Pathology, University of Kentucky, Lexington 40546-0091

ABSTRACT

Scibilia, K. L., and Shain, L. 1989. Protection of American chestnut with hypovirulent conidia of *Cryphonectria (Endothia) parasitica*. Plant Disease 73:840-843.

Cankers were initiated on American chestnut stems with mycelium of a virulent strain of *Cryphonectria (Endothia) parasitica* (Ep 155 = ATCC 38755) before or after application of conidia from isogenic strains that contained hypovirulence agents HI_2 from Italy (= strain Ep 780) or HT_2 from Tennessee (= strain Ep 905). Cankers initiated up to 10 wk after the application of conidia yielded cultures with hypovirulent morphology. Within 10 wk after spraying with Ep 780 conidia, 14 of 22 trees had at least one canker that produced hypovirulent inoculum as cirrhi. Conidia from Ep 780 converted significantly more cankers than did conidia from Ep 905. However, there was no significant difference in stem survival between conidial treatments after 1 yr.

American chestnut (*Castanea dentata* (Marsh.) Borkh.) was the most valuable timber tree in the eastern deciduous forest because of its abundance, regenerative capacity, rapid growth, and the utility of its wood and nuts (3,4,18,19). Chestnut blight, caused by *Cryphonectria (Endothia) parasitica* (Murr.) Barr, is directly responsible for the demise of American chestnut.

Hypovirulence, a reduction in the pathogenicity and sporulation of a pathogen, was closely associated with the decline of blight severity on European chestnut (*C. sativa* Mill.) in both Italy and France (7). In the *Cryphonectria* system, hypovirulence typically is associated with cytoplasmic double-stranded RNA that is transmissible among strains of similar vegetative compatibility (1,5,12).

The spread of hypovirulence in France has depended largely on the cooperation of farmers who inoculate cankers with hypovirulent strains provided by the biological control division in the Ministry of Agriculture (7). The inoculation of these trees is labor-intensive, approximately 10 min per tree, and expensive. Cost of inoculum production to treat 18,000 ha is about $300,000 per year (7). Unlike France, the United States has very few large trees in orchardlike settings that could make individual treatment practical. Attempts to control chestnut blight by hypovirulence in the United States have concentrated on more readily available smaller (3- to 10-cm-diameter) trees (9–11). The chances for controlling blight in the United States may be increased if hypovirulent inoculum could be applied economically in large amounts and if it had a protective as well as a curative effect.

Hypovirulent-appearing cultures were obtained from cankers initiated on dormant American chestnut stem segments with a virulent strain of the pathogen up to 3 wk after stems were sprayed with conidia of a hypovirulent strain (16). The following experiments were conducted to determine if conidia from hypovirulent strains could protect American chestnut trees from inoculations with a virulent strain under field conditions. A nontoxic agricultural sticker/extender also was tested to determine if it would increase the longevity of conidia on chestnut bark.

MATERIALS AND METHODS

General procedures. Cankers were initiated with virulent (V) strain Ep 155 (ATCC 38755) by removing a bark disk with a sterilized cork borer (6.5-mm diameter) and inserting into the wound a disk of mycelium and agar from the margin of a 5- to 8-day culture (10). Cultures were grown on potato-dextrose agar (Difco) amended with 100 mg/L of methionine and 1.0 mg/L of biotin (PDAmb) (1). Bark disks were replaced after inoculation to reduce desiccation. Both hypovirulent (H) strains were isogenic to Ep 155. Hypovirulent strain Ep 780 contains H agent HI_2 from Italy

Journal Series Paper 89-8-11-1 of the University of Kentucky Agricultural Experiment Station.

Accepted for publication 1 June 1989 (submitted for electronic processing).

and is moderately pathogenic. Hypovirulent strain Ep 905 contains H agent HT_2 from Tennessee and is slightly pathogenic (6). Hypovirulence agents were transmitted into conidia at frequencies of 43–77% for strain Ep 780 and 2–12% for strain Ep 905 (15). Conidial suspensions of hypovirulent strains were made by flooding 4- to 8-wk-old cultures with sterile double-deionized water. Final concentrations of conidia were determined with a hemacytometer and were obtained by diluting spore suspensions with sterile double-deionized water.

To test inoculations for hypovirulence, one or two bark disks were removed from the margin of each canker and placed in individual sterile, capped 1.5-ml plastic tubes. In the laboratory, individual bark disks were dipped in 70% ethanol and flamed 2–3 sec before placing them into PDAmb. Cultures were incubated at room temperature under fluorescent lights (16 hr/day, approximately 14 $\mu E \cdot m^{-2} \cdot s^{-1}$) for 6–8 days before identifying them as virulent or hypovirulent. Excised bark disks yielding a colony on PDAmb with typical H characteristics were taken as evidence for canker conversion. For Ep 780, H characteristics are white with a regular colony margin and a growth rate similar to EP 155; for EP 905, H characteristics are dark orange with an irregular colony margin and a growth rate about half that of Ep 155. Colonies of the parental V strain (Ep 155) were light orange with a regular margin. Additional evidence for conversion was obtained by pairing suspected H cultures with their isogenic virulent stock culture. Mycelial and agar disks, 4 mm in diameter, were placed 1 cm apart at the edge of 90 × 15 mm petri dishes containing cellophane-covered PDAmb. Paired strains were observed for 7–9 days for the transfer of H characteristics to Ep 155. Evidence for conversion, however, was not conclusive because the above strains are of the same genotype. To determine if conidia of Ep 780 are capable of converting rather than merely cohabiting in cankers with virulent strains, a separate test was conducted with several virulent strains with a nuclear marker. Four trees were inoculated with agar-mycelium disks of the following virulent strains with designated vegetative compatibility groups (Connecticut Agricultural Experiment Station): Ep 155 [40], Ep 287 [5], Ep 289 [71], Ep 360 [9], and Ep 516 [8]. With the exception of the first, our standard virulent strain, each is a methionine-requiring auxotroph. Inoculation wounds were sealed with latex caulk 1 wk later and painted with a conidial suspension of Ep 780 [40] at 2.1×10^7/ml. One bark disk from each canker was surface-sterilized and cultured in PDAmb 31 days after the application of conidia. Cultures were assessed for V or H morphology. Subcultures were grown on minimal media (14) with or without methionine to detect methionine-requiring genotypes.

Longevity of protection. Experiments were conducted on two sites to determine how long H conidia could protect American chestnut trees from blight. Trees on both sites were in the understory and showed no symptoms of blight. During June 1986, 26 trees at Black Mountain (37°57′0″ N, 82°51′30″ W; elevation approximately 1,100 m) were inoculated with V strain Ep 155 in a random cardinal direction at 30 cm above the ground. Two trees were inoculated similarly with sterile PDAmb. Three weeks after inoculation, these trees and 28 others received one of five spray treatments covering the entire bark surface from 30 to 130 cm above the ground. Pressurized 1.5-qt sprayers, sterilized with 70% ethanol and quadruple-rinsed with sterile water, were used to spray conidial suspensions on chestnut stems. Sprays were applied to the point of wetness; runoff was avoided. The treatments were: 1) sterile water, 2) conidia from Ep 780 (6.5×10^6/ml) or Ep 905 (6.3×10^6/ml) suspended in water alone; or 3) conidia suspended in water and Nu-film-17 (a nontoxic spreader/sticker/extender, Miller Chemical Corp., Hanover, PA) at the rate of one part Nu-film-17 to 1,600 parts conidial suspension. Additional cankers were initiated with Ep 155 in vertical alignment and 30 cm apart for a total of four inoculations per tree as follows: −3,1,2,3; −3,4,5,6; 6,1,2,3; or 6,4,5,6. Inoculation height is lowest on the left of each grouping and highest on the right. Each number represents the time of inoculation in weeks before (−) or after spray treatment.

Isolations from all cankers were made at both 2 and 4 wk after the final inoculation with Ep 155 (8 and 10 wk after spray). Two bark disks, one each from the top and bottom margins of each canker, were excised, surface-sterilized, and cultured on PDAmb. Because some cankers initiated 6 wk after spray with Ep 780 conidia yielded cultures typical of this H strain, we decided to reinoculate all Ep 780-sprayed trees with Ep 155 at 10 wk after spray treatment at 105 cm above the ground and opposite the original inoculations. These inoculations were sampled for hypovirulence 3 wk later. Cirrhi were collected from cankers 10 wk after conidial spray treatments to determine if any produced cultures typical of the H strains tested.

Hypovirulent cultures from the above inoculations at Black Mountain could have resulted from H inoculum arising from early inoculations that had been converted to H. To help ensure that conversion of inoculations with virulent mycelium was due to the H spray treatment, the following steps were taken in another test near McKee, Kentucky (37°26′0″ N, 84°01′30″ W; elevation approximately 400 m) in 1987. This site was located at least 40 km from the nearest known release of any H strain. A white H strain was used because few native white strains of *C. parasitica* exist in the United States. Inoculations with virulent mycelium were made only once.

Cultures of Ep 780, after growing for 10 days, were sealed with Parafilm to aid in sporulation. Final volumes of 500 ml contained sterile water, Ep 780 conidia in water only, or Ep 780 conidia in water amended with Nu-film-17 (1:1,600 or 1:160). Five trees were randomly assigned to each of the three conidial spray treatments and three trees to the sterile water control. Stems were sprayed from ground level to 180 cm above the ground. A severe rainstorm interrupted the experiment after 12 trees had been sprayed; 2 days later all trees were sprayed, most for a second time. Ten weeks after spraying, each tree received 15 inoculations with the V strain Ep 155. Inoculations were divided between either the north and south or the east and west faces, chosen at random. Inoculations began at 20 cm above the ground, were 20 cm apart, and were offset by 10 cm on opposing faces. Bark was not surface-sterilized before inoculation with Ep 155. Two bark disks from the lower and upper margins of each canker were cultured at 2 and 5 wk, respectively, after inoculation. Cultures were assessed as V or H as described above. Statistical analyses were by the chi-square test for goodness of fit. Expected frequencies were based on hypotheses intrinsic to the experimental design (17).

RESULTS

At Black Mountain, significantly ($P<0.01$) more inoculations made before or after trees were treated with conidia from Ep 780 yielded cultures with H morphology than similar inoculations treated with conidia from Ep 905 (Table 1). Some cankers initiated at each time before or after H conidial spray yielded cultures with H morphology. This includes 10 of the 24 trees inoculated 10 wk after being sprayed with Ep 780 conidia (*data not shown*). There were no significant trends between time of inoculation after H spray and apparent conversion. Frequency of recovery of cultures typical of Ep 905 was significantly greater ($P<0.05$) at 10 wk than at 8 wk after stems were sprayed with Ep 905 conidia. Of 69 isolates tested by in vitro conversion, 91% agreed with the initial cultural determination of H or V, i.e., isolates scored as H converted the V strain to H morphology in vitro.

Cankers treated with Ep 780 conidia suspended in water amended with Nu-film-17 yielded isolates with H morphology significantly more often

than did cankers treated with Ep 780 conidia in water only. This effect was seen in the first but not in the second isolation. The Nu-film-17 effect was insignificant when data from both isolations were pooled.

Cirrhi produced on cankers initiated on trees at Black Mountain produced cultures characteristic of Ep 780 or Ep 155. Cirrhi yielding cultures characteristic of Ep 905 were not detected. At 10 wk after spraying with H conidia, 14 of 22 Ep 780-treated trees had at least one canker producing Ep 780 inoculum in the form of cirrhi.

Twelve of 52 trees treated with H conidia still had not died back to the point of inoculation at the Black Mountain site 1 yr after inoculation. More of these were Ep 780-treated trees, but the difference between Ep 780- and Ep 905-treated trees was not significant. In fact, no treatment variables—i.e., diameter of trees, Ep 780 vs. Ep 905, addition of Nu-film-17 to the conidia-water suspension, and time of inoculation—were related significantly to survival. Only one of five Ep 780-treated trees that yielded cultures with Ep 780 morphology from all its inoculations with Ep 155 in 1986 was surviving in 1987. This comparison could not be made for Ep 905-treated trees because none of these yielded typical hypovirulent cultures from all cankers in 1986.

At the McKee site in 1987, some inoculations with virulent mycelium (Ep 155) made 10 wk after application of hypovirulent conidia (Ep 780) were converted to hypovirulence (Table 2). Conversion was not significantly affected by adding Nu-film-17 to conidial suspensions. Significantly more isolations made at 15 wk after the H treatments had hypovirulent morphology than isolations made at 12 wk. All except one of the 58 isolates tested by in vitro conversion agreed with cultural assessment of H or V.

At least one of the four inoculations intiated with virulent, methionine-requiring auxotrophs Ep 287, Ep 289, and Ep 360, which were sprayed with Ep 780 conidia, yielded a culture with Ep 780 characteristics. These H-appearing cultures grew only in the presence of methionine, suggesting strongly that Ep 780 was able to convert V strains of differing vegetative compatibility to hypovirulence.

Table 1. Conversion of cankers on American chestnut initiated by virulent *Cryphonectria parasitica* strain Ep 155 before (−) and after spraying stems with conidia from isogenic hypovirulent (H) strains Ep 780 and Ep 905

Inoculation time after H conidial spray (wk)	Converted cankers/total[x]			
	Ep 780-sprayed trees		Ep 905-sprayed trees	
	First isolation[y]	Second isolation	First isolation	Second isolation
−3	10/12	10/12	1/12	2/12
1	5/12	7/12	1/12	2/12
2	8/12	9/12	0/12	3/12
3	4/12	6/12	0/12	1/12
4	3/12	5/12	0/12	0/12
5	3/12	6/12	1/12	1/12
6	9/24	12/24	0/24	3/24
Total[z]	42/96 a	55/96 a	3/96 b	12/96 c

[x]Cankers yielding at least one isolate with H morphology were scored as converted. No significant differences in conversion were found between times after conidial spray by a chi-square test.
[y]First isolation at 8 wk and second isolation at 10 wk after trees were sprayed with conidial suspensions.
[z]Ratios followed by the same letter are not significantly ($P < 0.01$) different by a chi-square test.

Table 2. Conversion of cankers on American chestnut initiated by virulent *Cryphonectria parasitica* strain Ep 155 10 wk after spraying stems with conidia from isogenic hypovirulent (H) strain Ep 780

Spray mix[y]	Converted cankers/total[z]	
	Isolations 12 wk after spray	Isolations 15 wk after spray
Conidia + water	7/75	29/74
Conidia + Nu-film-17:water, 1:1,600	5/59	13/58
Conidia + Nu-film-17:water, 1:160	3/75	17/71
Sterile water	0/45	0/44

[y]Conidial suspensions contained 7.3×10^6 conidia per milliliter; sterile water contained no conidia. Each conidial treatment was applied to five randomly chosen stems; sterile water was applied to three stems. Significant differences in canker conversion were not observed among conidial treatments, as determined by a chi-square test.
[z]Cankers yielding at least one isolate with H morphology were scored as converted. Significantly ($P < 0.01$) more cankers were converted at 15 wk (5 wk after inoculation) than at 12 wk (2 wk after inoculation).

DISCUSSION

In a previous report (15), the margins of cankers intitiated by Ep 155 were inoculated in cork-borer wounds with disks of agar and mycelium of Ep 780 and Ep 905. Cultures with the typical hypovirulence morphology of these two strains were obtained from bark disks and cirrhi that were collected later from cankers. Hypovirulent conidia spray treatments have several possible advantages over the theraputic introduction of hypovirulent mycelium around cankers. These include ease of treatment, rapid treatment of large areas of bark (up to entire stems), and the ability to convert virulent strains for at least 10 wk after treatment.

These experiments were designed to test the principle of using conidia from hypovirulent strains to protect chestnuts from infection by the virulent pathogen. Conversion was encouraged for much of this study by using H and V strains that were isogenic, thereby avoiding vegetative incompatiblity between strains. Some inoculations may have been converted when they were initiated, since in both experiments the bark was not surface-sterilized before inoculation of cork-borer wounds. Conidia still must have survived on field-grown stems for 10 wk even if conversion occurred at the time cankers were initiated with the virulent strain. There was other evidence that H conidia can convert cankers through points other than the inoculation wounds: 1) Conversion apparently occurred when inoculations with virulent strains were sealed with latex caulk before application of H conidia and 2) naturally developed cankers were converted after application of H conidia (2).

The hypovirulence agents HI_2 and HT_2 differ substantially on their effect of recipient virulent strains. Strains with HI_2 (e.g., Ep 780) cause larger cankers (6), produce more cirrhi on cankers, and transfer their H agent to conidia more frequently than strains with HT_2 (e.g., Ep 905) (15). The present study indicates that conidia of Ep 780 are more efficient in converting inoculations with virulent strains than are conidia of Ep 905. Longer-term studies, however, would be useful to confirm or refute these results. An understanding of the effects of different H agents on virulent strains would be useful for the exploitation of hypovirulence as a biological control for chestnut blight.

Newly arising cankers caused by virulent wild types have been reported widely as a persistent problem in research plots (8,10,13). Trees with one or more converted cankers often are killed by these additional cankers. It may be possible to convert these newly arising cankers by treating the entire bole and branches with H conidia. If such treatment is effective against natural cankers for 10 wk, stands of American chestnut

could be protected with only two treatments per growing season. By this schedule, a first treatment applied in May presumably would convert many cankers that had developed up to this time. Protection from this treatment could last into the third month of the growing season. A second treatment, applied in August, could extend protection through the end of the growing season. Mixtures of conidia from H strains with a broad conversion capacity (12) should increase chances of success.

An increase in the number of cankers with hypovirulent strains was observed after several years of repeated treatments with either standard conversion procedures or hypovirulent spray treatments (2,9). Intensive treatment for 4–6 yr may be necessary to build a perpetuating hypovirulent reservoir in American chestnut stands. The ability of an H strain to colonize V cankers efficiently and the ability of converted cankers to produce sufficient H inoculum undoubtedly will affect the establishment and maintenance of this reservoir.

ACKNOWLEDGMENTS

We gratefully acknowledge the useful discussions with F. V. Hebard and the technical assistance of J. B. Miller.

LITERATURE CITED

1. Anagnostakis, S. L., and Day, P. R. 1979. Hypovirulence conversion in *Endothia parasitica*. Phytopathology 69:1226-1229.
2. Anagnostakis, S. L., and Kranz, J. 1987. Population dynamics of *Cryphonectria parasitica* in a mixed-hardwood forest in Connecticut. Phytopathology 77:751-754.
3. Anonymous. 1913. The chestnut tree—methods and specifications for the utilization of blighted chestnut. Pa. Chestnut Tree Blight Comm. Bull. 6. 16 pp.
4. Ashe, W. W. 1911. Chestnut in Tennessee. Tenn. Geol. Surv. Bull. 10-B. 35 pp.
5. 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.
6. Elliston, J. E. 1982. Effects of selected North American and Italian cytoplasmic hypovirulence agents on North American and Italian strains of *Endothia parasitica*. Pages 134-140 in: Proc. U.S. Dep. Agric. For. Serv. Am. Chestnut Coop. Meet. H. C. Smith and W. L. MacDonald, eds. 229 pp.
7. Grente, J. R., and Berthelay-Sauret, S. 1978. Biological control of chestnut blight in France. Pages 30-34 in: Proc. Am. Chestnut Symp. W. L. MacDonald, F. C. Cech, J. Luchoc, and C. Smith, eds. 122 pp.
8. Hebard, F. V., Griffin, G. J., and Elkins J. R. 1982. Summary research on biology of hypovirulent and virulent *Endothia parasitica* on blight-resistant and blight-susceptible chestnut trees at Virginia Polytechnic Institute and State University. Pages 184-192 in: Proc. U.S. Dep. Agric. For. Serv. Am. Chestnut Coop. Meet. H. C. Smith and W. L. MacDonald, eds. 229 pp.
9. Jaynes, R. A., and Depalma, N. K. 1982. Attempts to control chestnut blight with slurry and conidial sprays of hypovirulent strains. Pages 128-133 in: Proc. U.S. Dep. Agric. For. Serv. Am. Chestnut Coop. Meet. H. C. Smith and W. L. MacDonald, eds. 229 pp.
10. Jaynes, R. A., and Elliston, J. E. 1980. Pathogenicity and canker control by mixtures of hypovirulent strains of *Endothia parasitica* in American chestnut. Phytopathology 70:453-456.
11. Kuhlman, E. G. 1983. Effects of hypovirulence in *Cryphonectria parasitica* and of secondary blight infections on dieback of American chestnut trees. Phytopathology 73:1030-1034.
12. Kuhlman, E. G., Bhattacharyya, H., Nash, B. L., Double, M. L., and MacDonald, W. L. 1984. Identifying hypovirulent isolates of *Cryphonectria parasitica* with broad conversion capacity. Phytopathology 74:676-682.
13. MacDonald, W. L., Hindal, D. F., and Kaczmarczyk, W. J. 1982. Summary of *Endothia parasitica* hypovirulence research at West Virginia University. Pages 18-23 in: Proc. U.S. Dep. Agric. For. Serv. Am. Chestnut Coop. Meet. H. C. Smith and W. L. MacDonald, eds. 229 pp.
14. Puhalla, J. W., and Anagnostakis, S. L. 1971. Genetics and nutritional requirements of *Endothia parasitica*. Phytopathology 61:169-173.
15. Russin, J. S., and Shain, L. 1985. Disseminative fitness of *Endothia parasitica* containing different agents for cytoplasmic hypovirulence. Can. J. Bot. 63:54-57.
16. Scibilia, K. L., and Shain, L. 1986. Conversion of virulent chestnut blight cankers initiated after an application of hypovirulent conidia. (Abstr.) Phytopathology 76:1058.
17. Sokal, R. R., and Rohlf, J. 1969. Biometry. W. H. Freeman and Co., San Francisco, CA. 776 pp.
18. Sterling, E. A. 1901. Chestnut culture in the northeastern United States. Pages 87-114 in: Seventh Report to the State of New York Forest, Fish and Game Commission.
19. Zon, R. 1904. Chestnut in Southern Maryland. Bur. For. U.S. Dep. Agric. Bull. 53. 31 pp.