Effect of Eliminating dsRNA in Hypovirulent *Endothia parasitica*

D. W. Fulbright

Assistant professor, Department of Botany and Plant Pathology, Michigan State University, East Lansing 48824-1312.
Michigan Agricultural Experiment Station Journal Series Article 11136.
The author acknowledges the expert technical assistance of Alvin V. Ravenscroft.
Accepted for publication 9 February 1984.

ABSTRACT

Fulbright, D. W. 1984. Effect of eliminating dsRNA in hypovirulent *Endothia parasitica*. Phytopathology 74:722-724.

Hypovirulent isolates of *E. parasitica* were cured of detectable levels of double-stranded ribonucleic acid (dsRNA) by treatment with cycloheximide. Curing was accompanied by a dramatic increase in virulence, and by recovery of a normal colony morphology. Two isolates were resistant to cycloheximide-induced curing, but when their dsRNAs were transferred to other nuclear genetic backgrounds, curing was possible. All cured strains could be converted to the hypovirulent form by conversion with a hypovirulent strain. Converted strains gained dsRNA and displayed decreased virulence.

Hypovirulent isolates of *Endothia parasitica* (Murr.) Anderson have been found in populations of European and American chestnut trees (*Castanea sativa* Mill. and *C. dentata* [Marsh.] Borkh., respectively) infected with chestnut blight (3,13,15). These isolates are usually characterized as being less virulent than normal isolates of *E. parasitica*, have an abnormal morphology when grown on culture media, and contain double-stranded ribonucleic acid (dsRNA). In one strain the dsRNA has been associated with viruslike particles (6). These characteristics can be transmitted from hypovirulent strains to virulent strains following hyphal anastomosis (4,17). Hypovirulence in *E. parasitica* as described above has been referred to as "exclusive" (14), "cytoplasmic" (10), and "transmissible" hypovirulence (19).

Correlative evidence indicates that dsRNA is involved in hypovirulence, but this is still speculation since Koch's postulates have not been satisfied. The elimination of a virus or plasmid from a cell or organism with the concomitant loss of disease symptoms or phenotypic traits is one of the proofs required to determine if an extrachromosomal agent is responsible for phenotypic expression of that trait. Killer strains of *Saccharomyces cerevisiae* treated with cycloheximide lost the ability to inhibit sensitive strains of yeast (12). This loss has been attributed to the selective removal of a dsRNA segment associated with a viruslike particle called M in the cytoplasm of the killer strain of yeast (20).

Apparently the dsRNA in *E. parasitica* is stable even after the host has been subcloned several times (2); however, dsRNA-free cultures have been isolated from hypovirulent strains by culturing single conidia (8). The dsRNA-free cultures grew like normal virulent strains and were virulent when compared to normal strains (8,11,18). This report describes the curing of mycelial cultures of dsRNA by using cycloheximide.

MATERIALS AND METHODS

Curing experiments. Mycelial plugs from hypovirulent isolates were placed on curing media consisting of potato-dextrose agar (PDA, Difco) containing either 10 μg of cycloheximide per milliliter (PDA 10) or 20 μg of cycloheximide per milliliter (PDA 20). At 1- or 2-wk intervals, agar plugs were removed from the margins of the fungal colonies and placed on fresh PDA. Resulting colony morphology was compared to colony morphology from clones placed on PDA alone.

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.

©1984 The American Phytopathological Society

Pathogenicity tests. Isolates of *E. parasitica* were inoculated into 30-cm-long dormant woody branches of American chestnut (9). Wounds were made in the branches with a cork borer by removing a 4-mm-diameter plug of bark including tissue down to the vascular cambium. Mycelia were placed in the hole and covered with tape to prevent desiccation. Inoculated branch sections were placed in the dark in an incubator at 25 C. After 5 wk, the canker area was measured.

Alternatively, Golden Delicious apple fruits were inoculated by removing a 9 mm diameter × 7 mm deep plug of apple tissue (J. A. Dodds and J. E. Elliston, *personal communication*). Mycelia were placed in the wound and taped to prevent desiccation. The inoculated apples were placed in an open plastic bag and incubated at 25 C for 21 days. The resulting discolored area was measured and its area was calculated.

Conversions of virulent strains. Conversion of a virulent isolate to hypovirulent by a hypovirulent isolate was made by placing mycelial plugs of the virulent and the hypovirulent isolates ~2 mm apart at the edge of a petri dish containing PDA. Conversions were considered successful when the virulent isolate developed morphological characteristics of the hypovirulent isolate (1). Unpaired isolates served as controls. Presumptive converts were tested for virulence and the presence of dsRNA. Converted isolates were designated by listing the virulent isolate first followed in parentheses by the hypovirulent isolate that converted it. For example, in CL1(GHU4) the CL1 was the virulent isolate and GHU4 was the hypovirulent isolate that converted the virulent isolate.

dsRNA assays. Extraction of dsRNA was accomplished by modifications of the double-cellulose column procedure by Day et al (5), Morris and Dodds (16), and Dodds (7) except that fungal tissue was frozen in liquid N_2 and ground with glass beads in a chilled mortar and pestle. dsRNA was layered on polyacrylamide slab-gels (5%) and electrophoresed for 12 hr at 40 mA.

RESULTS

The virulent isolates CL1, CL2, CL3, and CL4 from a blighted American chestnut grove in Michigan are normal virulent isolates of *E. parasitica* that do not contain dsRNA (Table 1). Isolates GHU4, GH2, and RC-1 are morphologically abnormal in culture when compared to virulent strains, but are morphologically distinct when compared to each other. Furthermore, they contain three distinct banding patterns of dsRNA (Fig. 1, lanes A, C, and E) and exhibit reduced virulence on both dormant American chestnut wood and Golden Delicious apples when compared to the virulent strains (Table 1). Since pathogenicity tests on both chestnut wood and apple fruit gave the same relative results, pathogenicity tests on apple fruit were used for all remaining tests due to the availability of

material and shorter incubation time required.

Attempts to cure GHU4, GH2, and RC-1 were made by placing them on curing medium PDA 10. After 4 wk, only GHU4 grew as a normal isolate when transferred to fresh PDA while GH2 and RC-1 maintained abnormal culture morphology. This normal-appearing GHU4 culture was labeled GHU4pc10. Subsequent virulence tests and dsRNA assays showed this strain to be fully virulent (Table 2) and free of detectable dsRNA (Fig. 1, lane B). CL1 was treated as a control and usually maintained normal morphology and always maintained full virulence.

To test the possibility that dsRNA in GH2 and RC-1 was resistant to curing, the cured isolate GHU4pc10 was converted separately by GH2, RC-1 and GHU4. Conversion was verified by the virulence test and by observation of dsRNA in the converted strains. These strains, GHU4pc10(GH2), GHU4pc10(RC-1), and GHU4pc10(GHU4) were placed on PDA 10 or PDA 20. After 4 wk on curing media the cultures appeared morphologically normal and virulence had increased in every case (Table 2). Detectable levels of dsRNA were not found in these strains, indicating that the segments of dsRNA found in GH2, RC-1, and GHU4 were susceptible to curing, at least when harbored in the GHU4pc10 isolate (Fig. 1, lanes C, D, E, and F).

To determine if the curing of dsRNA was possible in genetic backgrounds other than GHU4, the virulent strains CL1, CL2, CL3, and CL4 were converted to hypovirulent by GHU4. CL1 was also converted by GH2 and RC-1 (Fig. 2, lanes A, B, and C). After the curing treatment CL1(RC-1)pc10 (Fig. 2, lane D), CL1(GHU4)pc20, CL3(GHU4)pc20, and CL4(GHU4)pc20 appeared morphologically normal, had increased virulence, and were dsRNA-free (Table 3). CL1(GH2) and CL2(GHU4) did not regain normal morphology after treatment with both PDA 10 and PDA 20 (*unpublished*). Normal morphology was observed in two isolates of CL1(GHU4)pc10; however, virulence did not increase to the expected level observed in the normal isolate CL1 (Tables 1 and 3). Assays for dsRNA in these isolates showed the presence of dsRNA segments.

DISCUSSION

An American hypovirulent isolate of *E. parasitica*, GHU4, obtained from a recovering tree in Grand Haven, MI, was cured of dsRNA by growing the isolate on PDA in the presence of cycloheximide. Two other hypovirulent isolates, GH2 and RC-1, representing different dsRNA banding patterns, were not cured. However, these dsRNA segments were cured once transferred to the GHU4 genetic background. The cured isolates were fully virulent and grew with normal morphology on PDA.

Although the mechanism of curing is unknown, Fink and Styles (12) postulated that since cycloheximide inhibits eukaryotic protein synthesis, cytoplasmic ribosomal protein synthesis must be necessary for the replication of the genetic determinant. This action could limit the genetic determinant and may cause it to be diluted as cell division progresses. This suggestion is supported by the observation that the dsRNAs are lost only when the hypovirulent strains were kept on a curing medium for 4 wk with mycelia being removed from the margin of the expanding colony.

When 10 μg of cycloheximide per milliliter failed to cause curing of dsRNA, 20 μg was tried. Higher concentrations of cycloheximide were required for curing of some isolates and GH2 dsRNA was only cured when in the GHU4pc10 nuclear background while CL2(GHU4), GH2, and RC-1 were resistant to curing. Higher concentrations were not tested due to the inhibitory effect on the fungus. The inability to cure dsRNA in two of the three native hypovirulent strains obtained from the field could be an

TABLE 1. Virulent and hypovirulent isolates of *Endothia parasitica* used in this study

Strain	Virulence tests[a] Apple fruit[b] (mm^2)	Chestnut wood[c] (mm^2)	dsRNA[d]
CL1	3,491	2,056	−
CL2	3,823	2,785	−
CL3	3,429	ND[e]	−
CL4	4,745	1,681	−
GHU4	218	99	+
GH2	1,452	618	+
RC-1	50	79	+

[a] Average of three replicates.
[b] Apple fruit were inoculated by removing a 9 × 7-mm-deep plug of apple tissue and inserting mycelia of strain to be tested. Three weeks later the area of the infected discolored region was calculated.
[c] Dormant chestnut wood was cut into 30-cm-long pieces. Wounds were made by removing a plug of bark down to the vascular cambium with a cork borer. Mycelia were placed in the hole. Area of infection was calculated 5 wk later.
[d] dsRNA was extracted by cellulose column procedures modified from Dodds (7).
[e] Not determined.

TABLE 2. Virulence of *Endothia parasitica* and presence of dsRNA in a cured hypovirulent isolate, GHU4pc10, converted with GHU4, GH2, and RC-1 dsRNA before and after curing treatment

Strain[a]	Virulence test[a] (mm^2)	dsRNA[c]
GHU4pc10	3,718	−
GHU4pc10(GHU4)	168	+
GHU4pc10(GHU4)pc20	3,672	−
GHU4pc10(GH2)	1,907	+
GHU4pc10(GH2)pc10	3,470	−
GHU4pc10(RC-1)	297	+
GHU4pc10(RC-1)pc10	3,145	−

[a] Apple fruit were inoculated by removing a 9 × 7-mm-deep plug of apple tissue and inserting mycelium of strain to be tested. Three weeks later the area of the infected discolored region was calculated.
[b] Isolates treated with curing medium and appearing normal were labeled as possible cured (pc) and labeled with the concentration of cycloheximide required for curing (10 or 20 μg of cycloheximide per milliliter). Parentheses indicate the hypovirulent isolate used to convert the cured strain GHU4pc10.
[c] dsRNA was extracted by cellulose column procedures modified from Dodds (7).

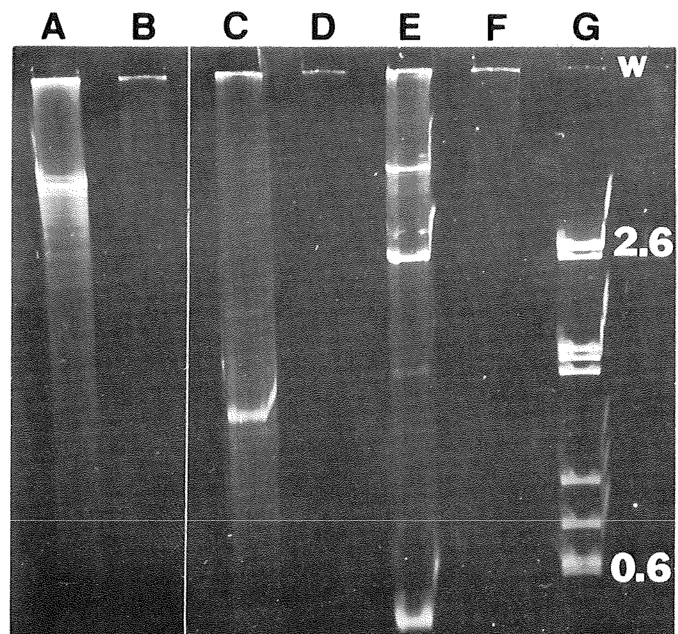

Fig. 1. Polyacrylamide gel electrophoresis of dsRNA isolated from *Endothia parasitica* strains GHU4, GHU4pc10 converted by RC-1 and GH2, and clones treated with curing media. **A**, GHU4; **B**, GHU4pc10; **C**, GHU4pc(RC-1); **D**, GHU4pc10(RC-1)pc10; **E**, GHU4pc10(GH2); **F**, GHU4pc10(GH2)pc10; and **G**, REO virus 3 genome used as molecular weight standards (×10^6). W = bottom of well.

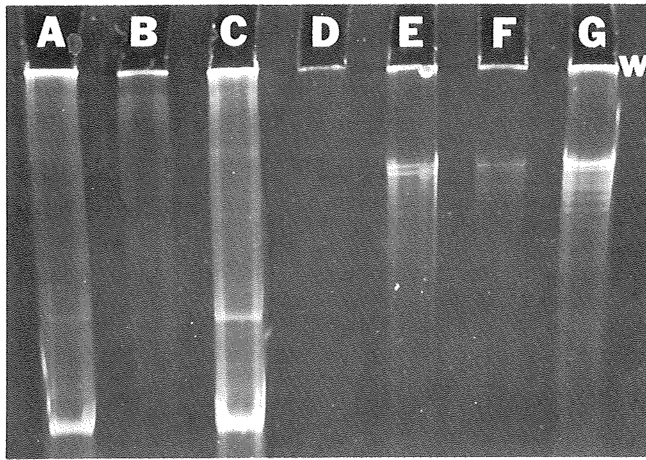

Fig. 2. Polyacrylamide gel electrophoresis of dsRNA isolated from *Endothia parasitica* strains CL1(RC-1), its cured clone CL1(RC-1)pc10, the converted strain CL1(GHU4), and the normal-appearing, intermediately virulent strains CL1(GHU4)pc10-1 and CL1(GHU4)pc10-2 obtained after growth on curing media. **A,** RC-1; **B,** CL1; **C,** CL1(RC-1); **D,** CL1(RC-1)pc10; **E,** CL1(GHU4); **F,** CL1(GHU4)pc10-1; and **G,** CL1(GHU4)pc10-2. W = bottom of well.

TABLE 3. Virulence and presence of dsRNA in virulent and converted isolates of *Endothia parasitica* before and after curing treatments

Strain[b]	Virulence tests[a] (mm^2)	dsRNA[c]
CL1(GHU4)	155	+
CL1(GHU4)pc10-1	1,115	+
CL1(GHU4)pc10-2	557	+
CL1(GHU4)pc20	2,778	−
CL1(RC-1)	371	+
CL1(RC-1)pc10	2,942	−
CL3(GHU4)	203	+
CL3(GHU4)pc20	3,631	−
CL4(GHU4)	167	+
CL4(GHU4)pc20	3,325	−

[a] Apple fruit were inoculated by removing a 9 × 7-mm-deep plug of apple tissue and inserting mycelium of strain to be tested. Three weeks later the area of the infected discolored region was calculated.

[b] Isolates treated with curing medium and appearing normal were labeled as possible cured (pc) and labeled with concentration of cycloheximide required for curing (10 or 20 μg/ml). Parentheses indicate the hypovirulent isolate used to convert the virulent strain.

[c] dsRNA was extracted by cellulose column procedures modified from Dodds (7).

indication of high dsRNA titer or nuclear factors governing general stability of the dsRNA molecules in these particular genetic backgrounds. In support of the latter possibility, all dsRNAs could be cured when introduced into the GHU4pc10 strain.

The ability to isolate morphologically normal CL1(GHU4)pc10 cultures containing dsRNA appears to indicate that dsRNA and abnormal morphology are not correlated. However, close examination of the dsRNA after electrophoresis shows that the banding pattern in the CL1(GHU4)pc10 cultures has been slightly modified (Fig. 2, lanes F and G). The top band in each case is brighter than the lower band. In CL1(GHU4), the two bands appear to be of equal intensity (Fig. 2, lane E). This might indicate that a change has taken place in the dsRNA species contained in the CL1(GHU4)pc10 cultures.

Although these results lend support to the argument that dsRNA is responsible for culture debilitation and hypovirulence, positive proof will only be obtained by infecting a normal strain with purified dsRNA or viruslike particles containing dsRNA.

LITERATURE CITED

1. Anagnostakis, S. L., and Day, P. R. 1979. Hypovirulence conversion in *Endothia parasitica*. Phytopathology 69:1226-1229.
2. Anagnostakis, S. L. 1981. Stability of double-stranded RNA components of *Endothia parasitica* through transfer and subculture. Exp. Mycol. 5:236-242.
3. Anagnostakis, S. L. 1982. Biological control of chestnut blight. Science 215:466-471.
4. Berthelay-Sauret, S. 1973. Utilisation de mutants auxotrophes dans les recherches sur le determinisme de "l'Hypovirulence exclusive." Ann. Phytopathol. 5:318.
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. Dodds, J. A. 1980. Association of type 1 viral-like dsRNA with club-shaped particles in hypovirulent strains of *Endothia parasitica*. Virology 107:1-12.
7. Dodds, J. A. 1980. Revised estimates of molecular weights of dsRNA segments in hypovirulent strains of *Endothia parasitica*. Phytopathology 70:1217-1220.
8. Dodds, J. A., and Elliston, J. E. 1978. Association between double-stranded RNA and hypovirulence in an American strain of *Endothia parasitica*. (Abstr.) Proc. 3rd Int. Cong. Plant Pathol., Munich, W. Germany.
9. Elliston, J. E. 1978. Pathogenicity and sporulation of normal and diseased strains of *Endothia parasitica* in American chestnust. Pages 95-100 in: Proceedings of the American Chestnut Symposium. W. L. MacDonald, F. C. Cech, J. Luchok, and C. Smith, eds. West Virginia University Press, Morgantown. 122 pp.
10. Elliston, J. E. 1981. Hypovirulence and chestnut blight research: Fighting disease with disease. J. For. 79:657-660.
11. Elliston, J. E. 1982. Hypovirulence in *Endothia parasitica* and suggested procedures for its detection and analysis. Pages 1-13 in: Proc. U.S. Dep. Agric. Forest Service Am. Chestnut Coop. Meeting. H. C. Smith and W. L. MacDonald, eds. West Virginia University Books, Morgantown. 229 pp.
12. Fink, G. R., and Styles, C. A. 1972. Curing of a killer factor in *Saccharomyces cerevisiae*. Proc. Nat. Acad. Sci. USA 69:2846-2849.
13. 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.
14. Grente, J., and Sauret, S. 1969. L'hypovirulence exclusive phénomène original en pathologie vegetale. C.R. Acad. Sci., Ser. D. 268:2347-2350.
15. Jaynes, R. A., and Elliston, J. E. 1982. Hypovirulent isolates of *Endothia parasitica* associated with large American chestnut trees. Plant Dis. 66:769-772.
16. Morris, T. J., and Dodds, J. A. 1979. Isolation and analysis of double-stranded RNA from virus-infected plant and fungal tissue. Phytopathology 69:854-858.
17. Van Alfen, N. K., Jaynes, R. A., Anagnostakis, S. L., and Day, P. R. 1975. Chestnut blight: Biological control by transmissible hypovirulence in *Endothia parasitica*. Science 189:890-891.
18. Van Alfen, N. K., Jaynes, R. A., and Bowman, J. T. 1978. Stability of *Endothia parasitica* hypovirulence in culture. Phytopathology 68:1075-1079.
19. Van Alfen, N. K. 1982. Biology and potential for disease control of hypovirulence of *Endothia parasitica*. Annu. Rev. Phytopathol. 20:349-362.
20. Young, T. W., and Yagiu, M. 1978. A comparison of the killer character in different yeasts and its classification. Antonie van Leeuwenhoek J. Microbiol. Serol. 44:59-77.