Double-stranded RNA in Endothia parasitica

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ABSTRACT

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Some strains of *Endothia parasitica* from Europe and North America contain dsRNA with a molecular weight of approximately 3.3×10^6. Species of dsRNA with lower molecular weights also were present in some of these strains. The dsRNA is transmitted by hyphal anastomosis. Transmission of dsRNA components to conidia, and to other strains by hyphal anastomosis, is not always complete.

Additional key words: hypovirulence, biological control, chestnut blight, cytoplasmic determinants.

Remnant rootstocks of native American chestnut, *Castanea dentata* Borkh., exist throughout eastern deciduous forests. Their sprouts may reach heights of 12.2-18.3 m (40-60 feet) before dying from blight infection. An effective self-propagating biological control to protect these trees could lead to rapid reestablishment over a wide area.

Experiments with abnormal strains of *Endothia parasitica* (Murr.) And., called hypovirulent (H), received from J. Grente in France (4, 5) have clearly shown potential for controlling chestnut blight in North America. These strains are incapable of sustained pathogenesis and carry a cytoplasmic determinant which renders them hypovirulent (4, 5, 6, 10). Virulent cankers that have been inoculated with H strains typically stop enlarging within several weeks. This is due to transfer of cytoplasmic determinants for hypovirulence (10). Wound-healing then proceeds by the formation of callus tissue at the canker margin.

Grente and Sauret (5) first suggested that hypovirulence is controlled by a transmissible cytoplasmic determinant. They showed that hypovirulent strains are unstable upon subculturing and suggested that progressive loss of cytoplasmic determinants is responsible for a range of phenotypes with white hypovirulent and stable, orange-pigmented virulent (normal) representing the extremes.

Bonifacio and Turchetti (2) examined many isolates from normal and healing cankers in Italy. They described similar morphological variation, but concluded that it is based on mutation and heterokaryosis and that hypovirulence results from the lowered fitness of heterokaryons. Van Alfen et al. (10) used a heterokaryon test to show that hypovirulence is cytoplasmically determined and that H strains control disease by transmitting hypovirulence to normal pathogenic strains by hyphal anastomosis. Moffitt and Lister (8) using a serological test found that two hypovirulent strains from France (originally designated JR2043 and B2025, K. J. Kessler *personal communication*) contained double-stranded (ds) RNA, whereas normal pathogenic strains from France (N2024) and Michigan did not. However, no dsRNA was detected by polyacrylamide gel electrophoresis of extracts prepared by homogenization in phenol and passage through a French press, nor were viruslike particles detected.

We report here the results of examining cultures from Europe and North America for dsRNA with a simplified extraction technique followed by polyacrylamide gel electrophoresis (J. A. Dodds, *unpublished*).

MATERIALS AND METHODS

Sources of strains.—Strains of *E. parasitica* were received from J. Grente in France and L. Mittempergher in Italy. North American strains were collected in Connecticut or sent to us by collaborators in other states.

Strains were tested for pathogenicity by inoculation alone into healthy stems of American chestnut and for hypovirulence by paired inoculation with one or more pathogenic strains. Methods of inoculation and criteria for judging pathogenicity have been described (1, 6, 10).

Cultures derived from single conidia, or hyphal tips, were classified as white or orange based on the degree of pigmentation after growth on Difco potato-dextrose agar (PDA) at room temperature (approximately 25 C) in light for 7 days.

Culture methods.—Stock cultures of *E. parasitica* were grown on PDA. Mycelia for extraction were grown in a liquid complete medium (9) modified by omission of glucose and addition of thiamine, 2 mg; potassium L-aspartate, 17.1 g; arginine, 0.1 g; and methionine 0.1 g per liter. The pH was adjusted to 4.0 with 1 N HCl. The liquid medium was dispensed in 100-ml aliquots in 946-ml (32-oz.) prescription bottles. After sterilization and

inoculation, the bottles were placed flat in an illuminated incubator at 28 C with a 16-hr photoperiod.

Nucleic acid extraction.—The mycelia were harvested after 7-9 days. The contents of five bottles were pooled, the pH was adjusted to 7.4 with 6 N NaOH, and centrifuged at 7,700 *g* for 10 min. The mycelium was resuspended in 100 ml 0.001 M Tris-HCl pH 7.4 buffer and centrifuged. The mycelial pad was pressed between layers of absorbent paper to remove buffer, weighed, suspended in 20 ml of 0.001 M Tris-HCl buffer containing 0.1% bentonite and homogenized in a Braun MSK homogenizer as described by Koltin and Day (7). Although a 4-min homogenization was used as a standard, 1 min was adequate. Mycelial pad weights ranged from 3.4 to 6.0 g. Following homogenization 0.5 ml of 25% sodium dodecyl sulfate and 20 ml of redistilled phenol saturated with 0.001 M Tris-HCl buffer were added to each homogenate. They were kept chilled in ice and shaken occasionally for 1 hr. The aqueous supernatant was removed after centrifugation at 7,700 *g* for 10 min, and two volumes of cold 95% ethanol were added to precipitate nucleic acids. Addition of sodium acetate (to 0.3 M concentration) prior to ethanol precipitation did not markedly improve recovery of dsRNA. After the extraction mixture had been chilled for at least 1 hr, the precipitate was collected by centrifugation, suspended in 10 ml TSE buffer (0.05 M Tris, 0.1 M NaCl, 0.001 M EDTA, pH 7.0) and dialyzed against TSE buffer for 2-3 hr at room temperature. After centrifugation (7,700 *g*, 10 min) the dialyzate was adjusted to 15% ethanol and passed through a Whatman cellulose CF 11 column (3) in a 20-ml disposable syringe barrel. The column bed (10 ml packed volume) was pre-washed with 50 ml TSE-15% ethanol and, after sample application washed again with 50 ml TSE-15% ethanol. The dsRNA retained by the column was eluted with two 5-ml aliquots of TSE-0% ethanol and was collected in 20 ml of cold 95% ethanol. Each column was used only once. After chilling for at least 1 hr, dsRNA was collected by centrifugation and dissolved in 0.5 ml electrophoresis buffer (.04 M Tris acetate, 0.001 M EDTA, pH 7.4) containing 20% glycerol. Samples were electrophoresed for 2-8 hr at 5-7 mA per gel in 5% polyacrylamide gels cast in 76 mm × 6 mm (i.d.) tubes. Following electrophoresis, gels were stained in methylene blue (2%) or toluidine blue. Approximate molecular weights were determined by co-electrophoresis with dsRNA from the P1 killer strain of *Ustilago maydis* (7).

RESULTS

Incidence of dsRNA.—Twenty-eight isolates of *E. parasitica* from France, Italy, and North America were tested for dsRNA. No dsRNA was found in any of the 15 pathogenic wild-type strains. However, dsRNA was found in 13 other strains including two from France, five from Italy, four from Michigan, and two from Virginia (Table 1, Fig. 1). None of these strains was as pathogenic as any of the 15 mentioned above (J. E. Elliston, *unpublished*). Two observations show that the method of extraction is reliable and that the results are repeatable. Frozen mycelial pads sampled and extracted on different occasions gave identical patterns. Stock 3 showed some variation in culture which we discuss below, however, more than six different mycelial pads, grown, extracted, and processed at different times, all gave the same three dsRNA bands after gel electrophoresis.

The six USA strains listed in Table 1 were collected from infected trees in Rockford, Michigan, and Alexandria, Virginia, and were all orange in color on PDA. One of them (strain 90) contained a unique species of dsRNA (approximate molecular weight 1.3×10^6). Tests for hypovirulence of these isolates are incomplete.

Transmission of dsRNA.—Finding dsRNA in the French H strains 3 and 4 prompted us to examine American H strains derived directly or indirectly from French strains by coinoculation in trees or by forcing heterokaryons on agar medium in the laboratory. The results are summarized in Table 2.

All derived strains which proved to be hypovirulent contained dsRNA, and the 3.3×10^6 component was common to all. The number of dsRNA components in a derived strain was either the same, fewer, or more than the number present in the donor strain from which it was derived. For example, the 3.2×10^6 component in strains 27, 43, and 36 was not present in strains 3, 9, or 14. However, as noted in Table 1 this component is present in strain 4, a single conidial isolate of strain 3. Strain 3 is the only strain with three dsRNA components; no strain derived from it contains all three.

Most authors working with hypovirulent strains of *E. parasitica* have reported instability and variation in morphology and hypovirulence. We examined cultures newly derived from single conidia of various strains. The source strains were predominantly white on PDA after 7 days of incubation in the light and single-conidial selections were either predominantly white or orange.

TABLE 1. Presence of dsRNA components in strains of *Endothia parasitica*

Source	Isolate nos.	Molecular wt.[a] 3.3	3.2	3.15	3.05	1.3
France	3 (B2025)	+[b]		+	+	
	4 (JR2043)	+	+			
Italy	49, 50, 51, 61, 66	+				
USA	60[c], 92[c], 93[c], 102, 103	+				
	90[c]	+				+

[a]Approximate molecular weight ($\times 10^6$).
[b]Indicates specific dsRNA component present.
[c]From Rockford, Michigan.

Most white selections showed some orange regions after 7 days of incubation in liquid medium for dsRNA isolation, suggesting segregation. The orange selections were more stable. The results of dsRNA extractions of these strains are summarized in Table 3. They show that a range of colony phenotypes with and without dsRNA arise from single conidia of dsRNA-containing strains. All of these strains with dsRNA contained the 3.3×10^6 component and were white in culture on PDA.

Endothia parasitica commonly produces perithecia and mature ascospores when the cankers mature in the fall. Several natural cankers that were showing signs of healing after inoculation with a white H strain, and which had perithecia, were selected for isolations. A total of 17 single-ascospore cultures were examined, representing 10 perithecia and three trees. All of the single-ascospore cultures were pathogenic and pigmented, and none carried dsRNA.

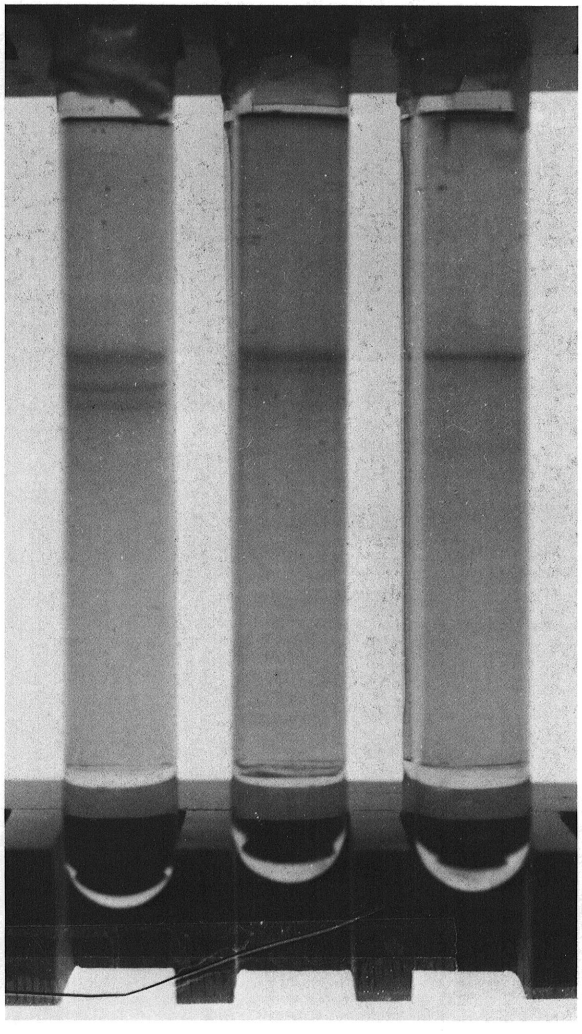

Fig. 1. Representative patterns of dsRNA components from strains of *Endothia parasitica* separated by electrophoresis on 5% polyacrylamide gels (7 hr) and stained with toluidine blue. Approximate molecular weights (all $\times 10^6$) were: left–3.3, 3.15, and 3.05; center–3.3 and 3.2; and right-3.3.

DISCUSSION

Van Alfen et al. (10) used a heterokaryon test to show that hypovirulence is cytoplasmically determined. The present experiments were undertaken to determine whether hypovirulence is always associated with the presence of dsRNA. This has so far proven to be true. Whether or not the dsRNA is associated with viruslike-particles is as yet undetermined. Transmission of dsRNA either through conidia or by means of hyphal anastomosis is not always exact or complete. For example, no strains derived from strain 3 by hyphal anastomosis carry all three of its dsRNA components. So far, transmission of dsRNA by ascospores has not been observed. Most perithecia produced in a healing canker arise in the central, older, region. These perithecia may be formed by islands of virulent mycelium within the healing canker.

It is not known whether the 3.3×10^6 MW dsRNA components present in the French, Italian, and native North American strains which contain dsRNA are identical. Most of the strains carrying dsRNA exhibit

TABLE 2. Presence of dsRNA components in American hypovirulent strains of *Endothia parasitica* derived from French strains[a]

Isolate	Hypovirulent source	Molecular Wt.[b] 3.3	3.2	3.15	3.05
9	3	+			
52	3	+			
14	9	+			
27	9	+	+		
43	9	+	+		
36	14	+	+		
53	4	+	+		

[a]Transfer of hypovirulence resulted from co-inoculations carried out in trees except for isolate 36 which came from a heterokaryon forced in culture. (Reported in Van Alfen et al., Science 189:890-891).

[b]Approximate molecular weight ($\times 10^6$).

TABLE 3. Phenotypes and dsRNA contents of single conidial isolates of *Endothia parasitica*

Sources	Single-conidial isolates	Phenotype on PDA	Presence of dsRNA
Italy (50)	50	white	+[a]
	66	white	+
	67	orange	−
Italy (51)	51	white	+
	64	white	+
	65	orange	−
Italy (49)	49	white	+
	63	white	+
	62	orange	−
USA (14)[b]	14	white	+
	98	orange	−

[a]Only the 3.3×10^6 component was present.

[b]Isolate 14 USA was derived from hypovirulent source strain 9.

various morphological abnormalities and are less pathogenic than the 15 wild-type strains that lack it (J. E. Elliston, *unpublished*). However, tests of these strains for hypovirulence are incomplete, and complicated by vegetative incompatibility (S. L. Anagnostakis, *unpublished*).

Differences between the three groups of strains carrying dsRNA also are apparent. The French strains and their derivatives generally carry more than one dsRNA component, Italian strains carry one, and native American strains carry one with the exception of #90 which also has a uniquely different component. Single spores of French strains, their derivatives, and Italian strains give rise to white and pigmented forms. The native American strains give rise only to pigmented forms. At present, no conclusion can be drawn about the relation between hypovirulence and the presence of dsRNA in *E. parasitica* beyond the fact that all confirmed H strains carry dsRNA.

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