Characteristics of dsRNA-Free and dsRNA-Containing Strains of *Endothia parasitica* in Relation to Hypovirulence

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Technical assistance by N. DePalma and B. Wooding and permission to use American chestnut on the property of E. Burke, Killingworth, CT, are gratefully acknowledged.
Accepted for publication 16 August 1984.

ABSTRACT

Elliston, J. E. 1985. Characteristics of dsRNA-free and dsRNA-containing strains of *Endothia parasitica* in relation to hypovirulence. Phytopathology 75:151-158.

DsRNA-free strains of *Endothia parasitica* and dsRNA-containing strains derived from abnormal chestnut blight cankers in France, Italy, and North America were compared to determine if simple reliable cultural indicators of dsRNA and low pathogenicity (hypovirulence [sensu stricto]) could be identified. The prototrophic dsRNA-free strains produced nearly identical colonies in culture, large lesions in apples, and large cankers with abundant stromata and spore tendrils in excised dormant American chestnut. Those tested in the field produced large cankers with abundant stromata and perithecia. In contrast, the dsRNA-containing strains all differed in culture from the dsRNA-free strains and from one another, all but one were deficient in pathogenicity and fruiting capacity, and their deficiencies spanned a wide range. Few produced perithecia. These observations suggest: simple, reliable cultural indicators of dsRNA and low pathogenicity probably do not exist, but overall appearance in culture may be a useful criterion for selecting strains to be tested for dsRNA; field tests spanning more than 6 mo are needed to detect the deficiencies of some dsRNA-containing strains; dsRNA is not invariably associated with subnormal pathogenicity and fruiting capacity in *E. parasitica*; a variety of factors may contribute to the diversity found among the dsRNA-containing strains and to the natural recovery phenomenon; and natural concepts and definitions of virulence and hypovirulence in *E. parasitica* are needed that accommodate this diversity. Broad concepts and operational definitions of virulence and hypovirulence (sensu lato), based on the pathogenicities and fruiting capacities of typical dsRNA-free strains (standards), are offered and discussed, and an approach is proposed for determining the factors which contribute to the hypovirulence (sensu lato) of individual strains.

Additional key words: biological control, *Castanea dentata, C. sativa, Malus sylvestris, Pyrus communis.*

Natural recovery of European chestnut, *Castanea sativa* Mill., from chestnut blight has been attributed to hypovirulence in the chestnut blight fungus, *Endothia parasitica* (Murr.) P. and H. And. (17,22,26). Although hypovirulence in *E. parasitica* has not been defined in the primary literature, weak pathogenicity (defined here as level of ability to colonize and kill host tissue), high curative capacity, whiteness in culture, and presence of dsRNA have been closely associated with it (5,9,18,19,29). These associations developed principally from studies of white strains isolated from abnormal cankers on recovering trees in southern France and northern Italy (18,19) and their white American derivatives (3,29). They sharply distinguish hypovirulent strains from typical strains of the fungus. The apparent constancy of these associations led to the use of whiteness as a diagnostic criterion of hypovirulence (28–30).

Because strains fitting this concept of hypovirulence contain transmissible cytoplasmic factors (6,29) that appear to be associated with dsRNA (9–11,23), one or more fungal viruslike agents have been assumed to be responsible for the hypovirulence and natural recovery phenomena.

A report from Italy (8), and preliminary studies of other dsRNA-containing strains from Italy and North America, cast doubt on the adequacy of the concept of hypovirulence based on the French strains. Results of these preliminary studies suggested that no consistent relations exist between colony color, level of pathogenicity, and presence of dsRNA. Because these dsRNA-containing strains were all obtained from abnormal cankers on recovering or persistent trees, these observations suggested that a wider range of strain types is involved in the natural recovery phenomenon and that a broader concept of hypovirulence is needed.

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The main objectives of this study were to compare the cultural characteristics, pathogenicities, and fruiting capacities of Italian and American dsRNA-containing strains, related dsRNA-free strains, and the French strains and their American derivatives to determine if any consistent cultural indicators of dsRNA or reduced pathogenicity could be found and to determine if a basis could be found for a natural concept and quantitative, operational definition of hypovirulence in this fungus. The possibility that apple fruits and trunk sections of dormant American chestnut (*C. dentata*) Borkh. might be useful for estimating the pathogenicity of *E. parasitica* in the laboratory during the winter also was explored. Preliminary reports of this work have been published (12,13).

MATERIALS AND METHODS

Table 1 lists pertinent data for 32 strains of *E. parasitica*. The French strains were provided by J. Grente, I.N.R.A., Clermont-Ferrand, France, and the four Italian mass isolates by L. Mittempergher, University of Florence, Florence, Italy. The American strains were isolated from bark samples provided by Mrs. R. D. Johnson, Rockford, MI; C. J. Reeder, Alexandria, VA; and G. Hicks, Valparaiso, IN. American strains containing dsRNA from the French strains and single conidial isolates (SCI) of the Italian strains also were used. Presence or absence of dsRNA was determined and reported by Day et al (9). Strains in which dsRNA was not detected are referred to as dsRNA-free.

The fungus was grown on 20 ml of Difco potato-dextrose agar amended with 100 mg of L-methionine and 0.1 mg of biotin per liter (PDAmb) in disposable 100 × 15-mm plastic petri dishes. Stock cultures were maintained on slants of PDAmb at 4 C. To minimize changes that often accompany serial transfers of dsRNA-containing strains, colonies for inoculum were grown directly from stock cultures. Plates were sealed with one layer of Parafilm® to minimize contamination, then inverted under fluorescent lights and incubated at 20 ± 2 C for 6–7 days with a 16-hr photoperiod (standard conditions). Inoculum consisted of 8-mm-diameter plugs

of agar cut with a flamed cork borer from the advancing edge of these colonies.

Cultural characteristics, growth in apples, and growth and asexual fruiting in excised dormant American chestnut stems were determined concurrently in an experiment first conducted in December and then repeated in January and March. To study cultural characteristics, two colonies of each strain were begun by gently pressing an inoculum plug, oriented with mycelium downward, onto the center of each plate and incubating them under standard conditions. Cultures were examined daily for the first 7 days and on the ninth and 12th days. Characteristics observed and recorded were: orientation and organization of leading mycelium; amount of aerial mycelium; colony color; presence, color, arrangement, and ornamentation of pycnidia and pycnidiumlike structures; and presence of spore masses. Organization of leading mycelium was rated according to a five-point scale ranging from 0 for disorganized growth to 4 for highly organized growth. Characteristics of pycnidia and leading mycelium were observed under a dissecting microscope at ×10–70.

Preliminary experiments with selected dsRNA-free and dsRNA-containing strains suggested that fruit of apples, *Malus sylvestris* Mill. (cultivars Golden Delicious, Red Delicious, Rome Beauty, and MacIntosh) and pears, *Pyrus communis* Linn. (cultivar Anjou), might be suitable substrates for estimating pathogenicity of *E. parasitica*. Because the soft brown lesions produced were most easily seen on Golden Delicious, this cultivar was used. Firm, mature apples were washed with tap water, then three 7-mm-diameter × 3–4-mm-deep plugs of tissue were removed with a flamed cork borer and spatula from points equally spaced around each fruit. Strains of *E. parasitica* were divided into groups of three, and each group was inoculated into each of five apples. Two inoculum plugs were inserted into each wound, mycelium facing inward, and pressed with a flamed spatula into complete contact with the tissue. Sites were covered with small pieces of masking tape to retard drying, then the apples were incubated in 31 × 24 × 11-cm plastic boxes under standard conditions. Lesion diameters were measured at 3- to 4-day intervals from the fifth to the 15th or 22nd day. Lesion areas were calculated from mean radii.

Tests of pathogenicity in dormant American chestnut were made with 118-cm-long × 5–10-cm-diameter trunk sections harvested in late November to early March. Trees were used that yielded three straight, relatively branch- and rhytidome-free sections. Branches were removed, cut ends and branch stubs were sealed with embedding paraffin (MP 53–55 C), and the bark surface was washed thoroughly with a pad of cheesecloth and tap water. Each strain was inoculated onto opposite sides of one trunk section from each of three trees. Where possible, successive pairs of inoculations were oriented 90 degrees to one another at 10- to 12-cm intervals along the stem to allow maximum tissue for colonization. Wounds made with a cork borer to the depth of the cambium were inoculated as described for apples. Inoculated trunk sections were incubated at 20 ± 2 C with a 16-hr photoperiod in 100 × 28 × 127-cm plastic-covered cases. High humidity was maintained with moist paper towels put on the bottom of the chambers and replaced at 3- to 4-day intervals. Cankers were measured and examined for stromata and spore tendrils at 3- to 4-day intervals beginning on the seventh or eighth day after inoculation and continuing for 5 wk. Canker area was calculated from canker length and width using the formula for an ellipse. The index of stromata occurrence was as follows: 0 = none; 1 = few; 2 = moderately abundant; 3 = abundant. Data from the 33rd day of the first, 27th day of the second, and the 21st day of the third experiment were used, because on these dates cankers produced by many of the dsRNA-free strains had attained comparable areas.

To determine the predictive value of laboratory pathogenicity tests and to assess capacity to produce perithecia, a representative group of pathogenic strains from the Italian and American collections was inoculated into American chestnut sprouts in a woodland in mid-June. Using procedures described above, each strain was inoculated into branch- and rhytidome-free regions of eight sprouts ranging from 4–12 cm dbh (diameter at 1.4 m above ground level). Each sprout was inoculated with four strains, one on each of four sides, at sites separated from each other along the trunk by approximately 30 cm. Cankers were measured, examined, and evaluated as before at monthly intervals from 1 to 6 and 9 to 13 mo after inoculation. Presence of perithecia was confirmed by dissecting stromata under a microscope. On each of the 9- to 13-mo observation dates, cankers were examined for signs that the fungus had invaded additional bark. Data were taken from the December (6-mo), April (10-mo), and July (13-mo) observations. The December data reflect the condition of the cankers at the end of the first growing season, when presence and abundance of stromata and perithecia could be assessed. This information on fruiting was confirmed by the 10- and 13-mo observations. The 13-mo data were chosen because it was clear by then which strains had invaded additional bark during the second season. This was also the last date when acceptably complete data could be obtained, because after July many trees succumbed to infections by normal strains, or to natural infections that were not evident at the start of the experiment. The statistical significance of differences in colony, lesion, and canker areas was determined by using Duncan's new multiple range test, $P = 0.05$.

TABLE 1. Pertinent information for strains of *Endothia parasitica* studied

Strain	ATCC number	Geographic origin	dsRNA[a]	Isolate type, source	Donor of dsRNA
French and French-derived American strains					
EP-2		France	−	MMI[b]	−[c]
EP-3	38770	France	+	MMI	−
EP-4	38769	France	+	SCI[d],EP-3	EP-3
EP-6	22508	Connecticut	−	LIM[e],*met*[f]	−
EP-14	38768	Connecticut	+	MMI,EP-6,*met*	EP-3
EP-98		Connecticut	−	SCI,EP-14,*met*	−
EP-42	38751	Connecticut	−	MMI	−
EP-43	38767	Connecticut	+	MMI,EP-42	EP-3
EP-27		Connecticut	+	MMI,EP-42	EP-3
EP-52		Connecticut	+	MMI	EP-3
EP-53	38766	Connecticut	+	MMI	EP-4
Italian strains					
EP-46		Tuscany, Italy	−	MMI	−
EP-62		Tuscany, Italy	−	SCI,EP-49	−
EP-49	39759	Tuscany, Italy	+	MMI	−
EP-63		Tuscany, Italy	+	SCI,EP-49	EP-49
EP-94	38763	Tuscany, Italy	+	MMI,EP-49	EP-49
EP-95		Tuscany, Italy	+	MMI,EP-49	EP-49
EP-67	38753	Tuscany, Italy	−	SCI,EP-50	−
EP-50		Tuscany, Italy	+	MMI	−
EP-66		Tuscany, Italy	+	SCI,EP-50	EP-50
EP-65		Tuscany, Italy	−	SCI,EP-51	−
EP-51	38758	Tuscany, Italy	+	MMI	−
EP-64		Tuscany, Italy	+	SCI,EP-51	EP-51
American strains					
EP-29	38754	Connecticut	−	MAI[g]	−
EP-60	38765	Michigan	+	MMI	−
EP-88	38757	Michigan	+	MMI	−
EP-89		Michigan	−	MMI	−
EP-90	38764	Michigan	+	MMI	−
EP-91		Michigan	−	MMI	−
EP-99		Indiana	−	MMI	−
EP-102		Virginia	+	MMI	−
EP-103		Virginia	+	MMI	−

[a] Presence of dsRNA determined by Day et al (9).
[b] Mass mycelial isolate.
[c] Hyphens in the last column indicate that the donor of dsRNA, if any, is unknown.
[d] Single conidial isolate.
[e] Laboratory-induced mutant.
[f] Methionine-requiring.
[g] Mass ascospore isolate.

RESULTS

French and French-derived American strains. *Cultural characteristics.* The French, French-derived American, and related

strains were separable into three groups: dsRNA-free, orange strains (EP-2, 6, 42, and 98); dsRNA-containing, predominantly white strains (EP-3, 14, 27, 43, 52, and 53); and a dsRNA-containing dark orange-brown strain (EP-4) (Figs. 1 and 2).

The dsRNA-free strains were nearly indistinguishable. They grew rapidly and produced abundant aerial mycelium, particularly in the region immediately behind the colorless, highly organized advancing mycelium (degree of organization = 3.5–3.8). Only these strains produced the orange color typical of *E. parasitica*. The pigmentation appeared 3–4 days after transfer and was correlated with differentiation of pycnidia. Pycnidia became more or less smooth, hemispherical, and dark orange when mature and were produced more or less separately on the mycelium in well-defined concentric rings that corresponded with photoperiods. Occasionally, EP-6 and EP-98, methionine-requiring auxotrophs, produced pycnidia in radially elongated aggregates. Pycnidia began to release orange spore masses when colonies were 5–7 days old.

The predominantly white dsRNA-containing strains differed widely among themselves, but were easily distinguished from dsRNA-free strains. Only EP-3, 27, and 52 grew as rapidly as dsRNA-free strains. Leading mycelium was less organized (degree of organization = 1.5–2.8), and aerial mycelium was less abundant. Strain EP-3 remained white through the ninth day. Strains EP-14, 27, 43, and 52 developed yellowish centers beginning 5–6 days after transfer. This color was associated with mixtures of white and light to dark yellow pycnidia that were smaller than those produced by dsRNA-free strains, scattered on the mycelium, and ornamented with surface hyphae. Only EP-3 did not produce pycnidia, and only pycnidia of EP-43 liberated spore masses, beginning 9–12 days after transfer.

Strain EP-4, the only heavily pigmented dsRNA-containing French strain, grew more slowly than all other strains. Advancing mycelium was sparse and disorganized (degree of organization = 0.3), and aerial mycelium (when present) consisted of short, fine hyphae thinly distributed over the colony surface. Minute pycnidiumlike structures developed in chainlike arrangements on the mycelium beginning a short distance behind the leading edge. Spore masses were not detected.

Growth in apples. These strains differed widely in ability to grow in apples (Fig. 2A), and none produced fruiting bodies. The two prototrophic dsRNA-free strains produced identical large, brown, sunken lesions. The two methionine-requiring, dsRNA-free strains and all dsRNA-containing strains produced significantly smaller lesions, with some significant differences between strains. Strains EP-4 and 14 were nonpathogenic. The strains that grew in apple continued to grow for the duration of the experiments.

Growth and asexual fruiting in dormant American chestnut stems. The dsRNA-free strains produced large cankers, many stromata, and spore tendrils (Fig. 2B, Table 2). Cankers produced by three of the dsRNA-free strains did not differ significantly in area or abundance of stromata. Production of spore tendrils was less consistent. All dsRNA-containing strains were significantly less pathogenic, and none produced stromata or spore tendrils. All except EP-4 and 14 colonized a small amount of bark but stopped growing within 8 days after inoculation.

Italian strains. *Cultural characteristics.* The Italian strains were separable into two groups: dsRNA-free, orange strains, and dsRNA-containing, predominantly white strains (Fig. 3). The dsRNA-free strains were indistinguishable from those in the French and French-derived American collections. Colonies of the dsRNA-containing strains had diverse abnormalities which differed from those of the French and French-derived American strains. All were easily distinguished from the dsRNA-free strains. Strains EP-49, 51, and 64 grew rapidly and produced abundant

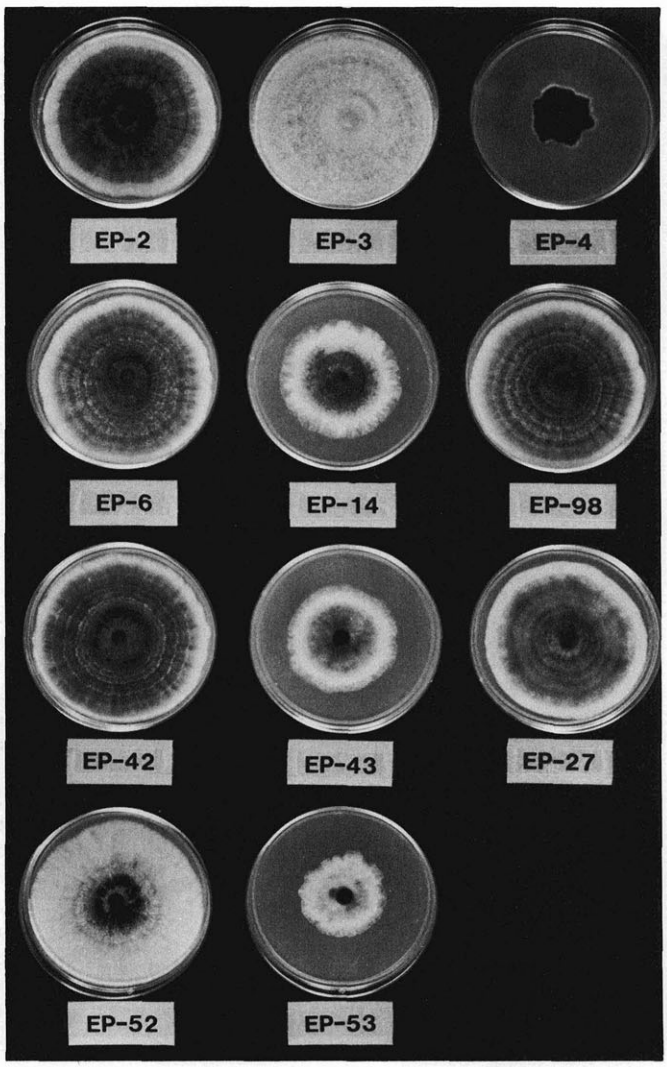

Fig. 1. French and French-derived American dsRNA-free and dsRNA-containing strains of *Endothia parasitica* grown 7 days at 20 C with a 16-hr photoperiod. Strains EP-2, 6, 98, and 42 were dsRNA-free, and EP-3, 4, 14, 43, 27, 52, and 53 were dsRNA-containing.

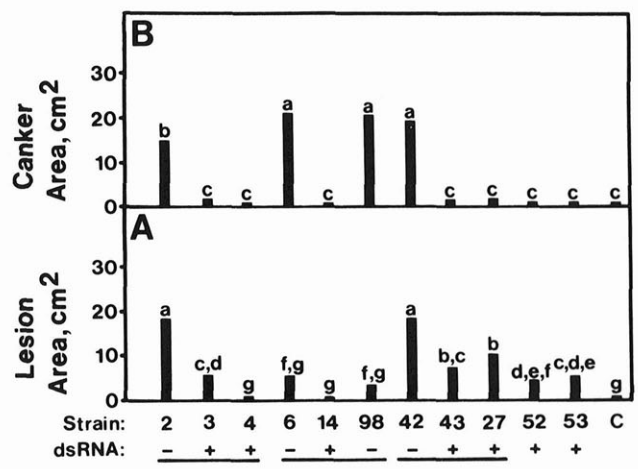

Fig. 2. Growth of French and French-derived American dsRNA-free and dsRNA-containing strains of *Endothia parasitica* in Golden Delicious apples and excised dormant American chestnut stems in the laboratory. **A,** Mean lesion area (square centimeters) 15 days after inoculation of Golden Delicious apples. Means are for 10 lesions per strain, five in each of two experiments. **B,** Mean canker area (square centimeters) in dormant American chestnut stems. Means are for 18 cankers per strain, six in each of three experiments. Data were taken from the 33rd, 27th, and 21st days of the first, second, and third experiments, respectively. Inoculated apples and stems were incubated at 20 C with a 16-hr photoperiod. Within a graph, means headed by the same letter are not significantly different according to Duncan's new multiple range test, $P = 0.05$. Symbols: C, PDAmb control substituted for inoculum; −, dsRNA-free; and +, dsRNA-containing. Strains sharing a common underline have the same genetic background.

aerial mycelium, but they had weakly organized leading mycelium (degree of organization = 0.8–2.3). The others, EP-50, 63, 66, 94, and 95, grew much more slowly, produced little aerial mycelium, and had disorganized leading mycelium. All of the dsRNA-containing strains remained white for at least the first 5 days after transfer, then some developed cream, yellow, or light orange centers, or light orange concentric rings. Strain 64 remained white for the duration of the experiments, and strain 66 developed a dark yellowish-brown center. Pigmentation was associated first with developing pycnidia then also developed in the surrounding mycelium. The dsRNA-containing strains produced diverse pycnidiumlike structures. The fast-growing strains (EP-49, 51, and 64) produced abnormally large structures with yellow and orange apices and hyaline surface hyphae radiating from them. Strain 66 produced minute white, yellow, and orange pycnidia. The arrangement of pycnidia and pycnidiumlike structures varied from random (strains EP-50, 63, 66, 94, and 95) to weakly organized concentric rings (EP-49 and 51). Pycnidia of three strains (EP-49, 63, and 94) released spore masses 7–12 days after transfer.

Growth in apples. The four dsRNA-free strains and four of the dsRNA-containing strains (EP-49, 61, 63, and 64) produced similar large lesions in Golden Delicious apples (Fig. 4A). The other dsRNA-containing strains produced significantly smaller lesions and differed significantly among themselves in this capacity. All strains grew continuously for the duration of the experiments.

Growth and asexual fruiting in dormant American chestnut stems. The four dsRNA-free strains and two of the fast-growing dsRNA-containing strains (EP-49 and 64) produced similarly large cankers and abundant stromata in excised dormant chestnut (Fig. 4B, Table 2). All except strain 64 also produced abundant spore tendrils. Three dsRNA-containing strains (EP-66, 94, and 95) were nearly nonpathogenic, and only one (EP-66) produced stromata and spore tendrils. Two strains (EP-94 and 95) stopped growing within 15 days after inoculation. The remaining dsRNA-containing strains had intermediate levels of pathogenicity and asexual fruiting capacity in dormant chestnut and grew for the duration of the experiments.

Growth and fruiting in American chestnut sprouts in the field. Of the seven strains tested in the field, only the dsRNA-free strain (EP-46) and one of the fast-growing dsRNA-containing strains (EP-49) sustained pathogenesis during the first 6 mo. These two strains produced similarly large cankers with abundant stromata, but only the dsRNA-free strain produced perithecia (Fig. 4C, and Table 2); these were first evident in August and were abundant by December. During this period, the other dsRNA-containing strains produced small cankers with stromata but no perithecia. A few perithecia developed in cankers produced by EP-63 and were first detected 9 mo after inoculation. During the second growing season, only the dsRNA-free strain resumed growth. The apparent increase in canker area for other strains was due to callus development and radial growth of the trees.

All infections killed bark tissue to the vascular cambium.

TABLE 2. Production of stromata, spore tendrils, and perithecia on American chestnut

		Laboratory[a]			Field[b]			
Strain	dsRNA	Cankers with stromata (%)	Abundance of stromata[c]	Cankers with tendrils (%)	Cankers with stromata (%)	Abundance of stromata[c]	Cankers with perithecia (%)	Abundance of perithecia[c]
French and French-derived American strains								
EP-2	−	89	2.1	78	...[d]	...	...	...
EP-3	+	0	0	0	...	...	...	...
EP-4	+	0	0	0	...	...	...	...
EP-6[e]	−	83	2.3	56	...	...	...	...
EP-14[e]	+	0	0	0	...	...	...	...
EP-98[e]	−	56	1.2	11	...	...	...	...
EP-42	−	100	2.3	100	...	...	...	...
EP-43	+	0	0	0	...	...	...	...
EP-27	+	0	0	0	...	...	...	...
EP-52	+	0	0	0	...	...	...	...
EP-53	+	0	0	0	...	...	...	...
Italian strains								
EP-46	−	83	1.8	83	100	2.6	100	2.75
EP-62	−	100	1.9	100	...	...	...	...
EP-49	+	89	1.5	89	100	1.1	0	0
EP-63	+	39	0.8	39	50	0.5	50	0.5
EP-94	+	0	0	0	...	...	...	...
EP-95	+	0	0	0	...	...	...	...
EP-67	−	100	2.3	100	...	...	...	...
EP-50	+	100	1.6	100	88	1.1	0	0
EP-66	+	44	0.9	17	75	0.9	0	0
EP-65	−	100	2.2	100	...	...	...	...
EP-51	+	89	1.1	78	100	1.2	0	0
EP-64	+	83	1.6	33	100	1.5	0	0
American strains								
EP-29	−	100	2.7	94	100	3.0	100	3.0
EP-60	+	0	0	0	...	...	...	...
EP-88	+	89	1.7	50	100	2.5	0	0
EP-89	−	100	1.9	100	...	...	...	...
EP-90	+	58	0.6	8	88	1.5	0	0
EP-91	−	100	2.3	100	...	...	...	...
EP-99	−	94	2.2	94	...	...	...	...
EP-102	+	11	0.2	0	88	1.7	63	0.9
EP-103	+	72	1.9	61	100	2.7	100	2.7

[a] Eighteen cankers, six in each of three experiments, were examined per strain. Observations were made on the 33rd, 27th, and 21st days of the first, second, and third experiments, respectively.

[b] Eight cankers per strain were examined 6 mo after inoculation.

[c] Abundance of stromata or perithecia was rated on a four-point scale: 0, none; 1, few; 2, moderately abundant; and 3, abundant. Values are means.

[d] Not tested.

[e] Methionine-requiring auxotroph.

Containment of the dsRNA-containing strains was accompanied or followed by development of callus tissue at the canker margin. As this regenerated bark and sapwood enlarged radially and tangentially, the infected bark separated from the sapwood, split, gradually disintegrated, and fell away.

American Strains. *Cultural characteristics.* All strains in this collection produced pigmented colonies (Fig. 5). The dsRNA-free strains (EP-29, 89, 91, and 99) were indistinguishable from those in the other collections. Pigmentation of the dsRNA-containing strains developed 3–5 days after transfer and ranged from yellow-brown (EP-60) to orange (EP-103) and dark orange-brown (EP-88, 90, and 102).

Abnormalities of each dsRNA-containing strain were different, but only three strains (EP-60, 90, and 102) were distinguished easily from dsRNA-free strains. The only abnormality common to the dsRNA-containing strains was absence of distinct concentric rings of pycnidia, and only EP-103 was radially symmetric. Strains 60, 90, and 102 had poorly organized leading mycelium (degree of organization = 0.0–0.8). Strains 60 and 90 grew slowly, had little aerial mycelium, and produced minute, dark-brown pycnidia in tightly packed masses on the oldest mycelium and in chainlike aggregates nearer the margin. Strains 88 and 103 had highly organized leading mycelium (degree of organization = 3.5–3.8). Strain 88 differed from dsRNA-free strains in its darker pigmentation, irregular margin, and radial aggregates of pycnidia. Strain 103 most closely resembled dsRNA-free strains.

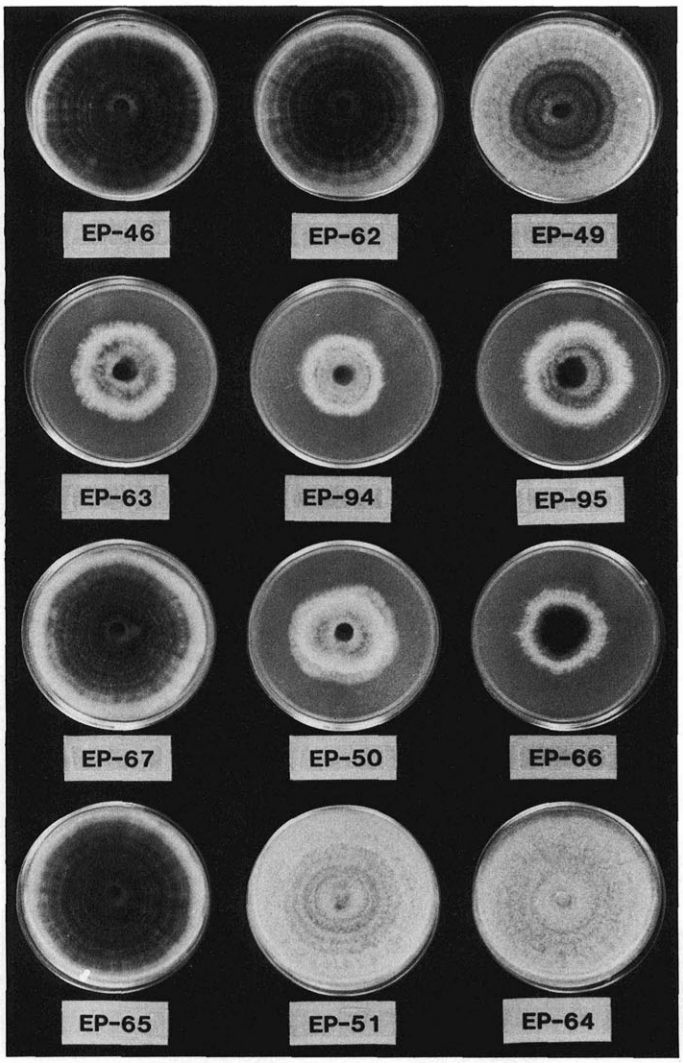

Fig. 3. Italian dsRNA-free and dsRNA-containing strains of *Endothia parasitica* grown 7 days at 20 C with a 16-hr photoperiod. Strains EP-46, 62, 67, and 65 were dsRNA-free, and EP-49, 63, 94, 95, 50, 66, 51, and 64 were dsRNA-containing.

Growth in apples. The four dsRNA-free strains and strain 103 produced similarly large lesions in Golden Delicious apples (Fig. 6A). Strain 60 was nonpathogenic, strain 90 produced small lesions, and strains 88 and 102 produced intermediate-sized lesions. The strains that grew in apple continued to grow for the duration of the experiments.

Growth and fruiting in dormant American chestnut stems. Although all four dsRNA-free American strains produced large cankers, abundant stromata, and spore tendrils, the cankers differed significantly in area (Fig. 6B, and Table 2). The least pathogenic dsRNA-free strains (EP-91 and 99) and the most pathogenic dsRNA-containing strains (EP-88 and 103) produced similar cankers, but the dsRNA-free strains produced more spore tendrils. The other dsRNA-containing strains either were nonpathogenic (EP-60) or produced small cankers with little fruiting (EP-90 and 102). The strains that grew in dormant chestnut continued to grow for the duration of the experiments.

Growth and fruiting in American chestnut sprouts in the field. All five strains tested in the field sustained pathogenesis during the first 6 mo after inoculation, but three of the dsRNA-containing

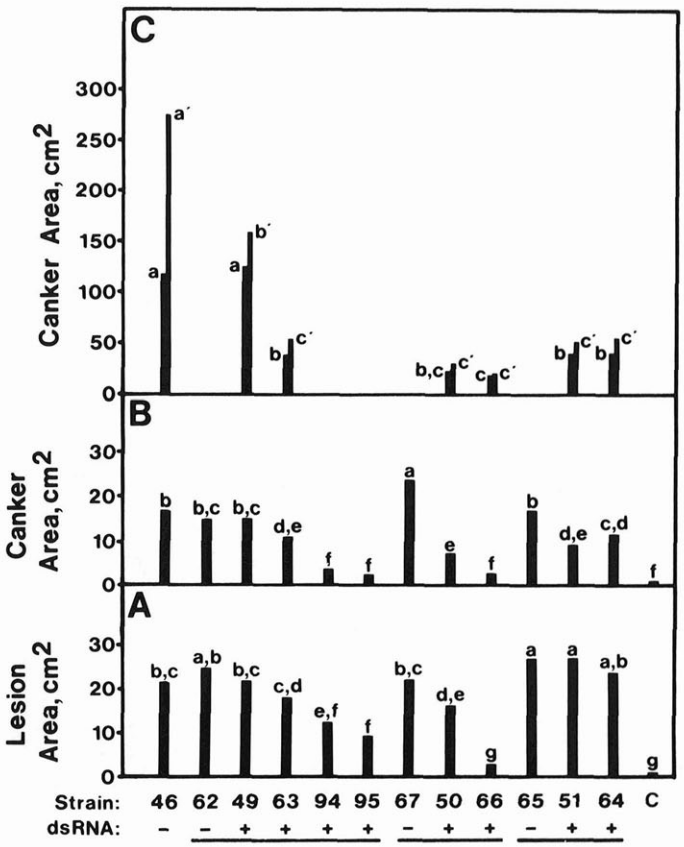

Fig. 4. Growth of Italian dsRNA-free and dsRNA-containing strains of *Endothia parasitica* in Golden Delicious apples, excised dormant American chestnut stems, and American chestnut sprouts in the field. **A,** Mean lesion area (square centimeters) 15 days after inoculation of Golden Delicious apples. Means are for 10 lesions per strain, five in each of two experiments. **B,** Mean canker area (square centimeters) in dormant American chestnut stems. Means were calculated for 18 cankers per strain, six in each of three experiments. Data were taken from the 33rd, 27th, and 21st days of the first, second, and third experiments, respectively. **C,** Mean canker area (square centimeters) 6 and 13 mo after inoculation into sprouts of American chestnut in the field. Means are for eight cankers per strain. Inoculated apples and dormant American chestnut were incubated at 20 C with a 16-hr photoperiod. Within a graph, means headed by the same letter are not significantly different according to Duncan's new multiple range test, $P = 0.05$. In graph C, letters without an apostrophe are for 6-mo canker areas, and those with an apostrophe are for 13-mo areas. Symbols: C, PDAmb control substituted for inoculum; −, dsRNA-free; and +, dsRNA-containing. Strains sharing a common underline have the same genetic background.

strains (EP-88, 90, and 102) grew slowly (Fig. 6C). The dsRNA-free strain (EP-29) and the least abnormal dsRNA-containing strain (EP-103) produced large cankers and abundant stromata and perithecia. Of the other dsRNA-containing strains, only EP-102 produced perithecia, but these were not numerous. During the second growing season, all strains except EP-88 colonized additional tissue. Strains EP-29 and 103 grew most actively, producing large cankers which were indistinguishable from one another.

DISCUSSION

In 1917, Shear et al (24) reported striking uniformity among isolates of *E. parasitica* from eastern North America, British Columbia, and China, and they noted that this uniformity was maintained through hundreds of generations. The close similarity found among the prototrophic dsRNA-free strains used in this study suggests that this uniformity has persisted. If so, it provides a basis for defining normalcy in *E. parasitica* operationally, ie, a representative set of dsRNA-free strains can be used in experiments as standards to define normal cultural characteristics and normal levels of pathogenicity and fruiting capacity. Presumably, the minor deviations found in dsRNA-free strains EP-6 and 98 are associated with their auxotrophy.

The diversity found in the cultural characteristics, pathogenicities, and fruiting capacities of the dsRNA-containing strains contrasts sharply with the uniformity of the dsRNA-free strains. In culture, all of the dsRNA-containing strains differed from the dsRNA-free strains and from one another, although some differences were minor. No abnormal character was common to them all. Thus, overall appearance in culture, rather than any one cultural character, may be a useful indicator of dsRNA, or at least it may be a useful criterion for selecting strains to be tested for dsRNA.

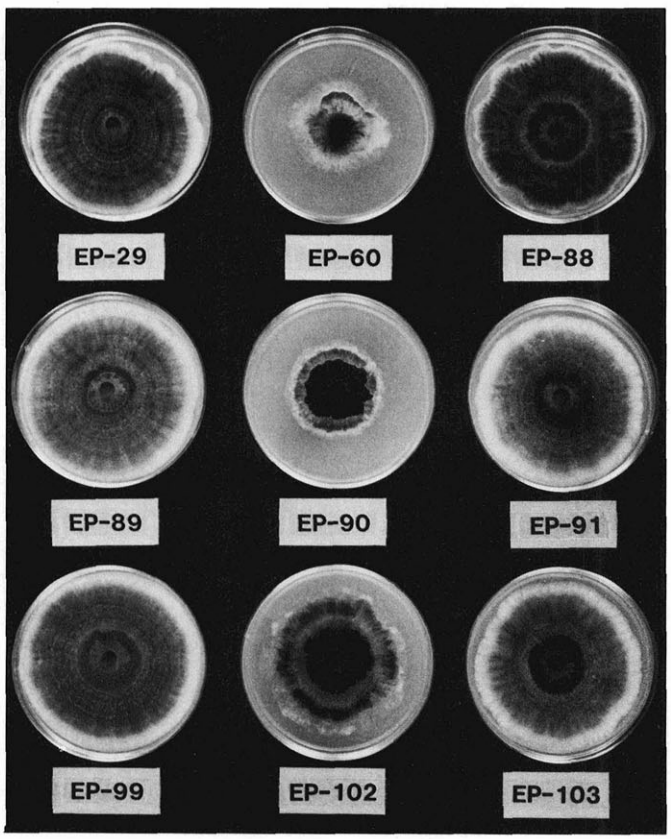

Fig. 5. American dsRNA-free and dsRNA-containing strains of *Endothia parasitica* grown for 7 days at 20 C with a 16-hr photoperiod. Strains EP-29, 89, 91, and 99 were dsRNA-free, and strains EP-60, 88, 90, 102, and 103 were dsRNA-containing.

All of the dsRNA-containing strains except EP-103 were deficient in pathogenicity compared with the dsRNA-free strains. These deficiencies spanned a wide range and were manifested in several ways. For example, a few strains were nonpathogenic in apple fruit and in chestnut bark. Some colonized a small amount of chestnut bark during the first week or two after inoculation and then stopped, but in apple fruit they grew slowly but continuously. Others colonized significant amounts of tissue in all three tests, and stopped only in the field test, several months after inoculation. Other strains colonized host tissue slowly but continuously in all three tests, except during the winter period of the field test. Finally, the pathogenicity of one strain, EP-49, was equivalent to that of the dsRNA-free strains for the duration of the laboratory tests and the first growing season of the field test. Its deficiency only became apparent during the second growing season. This strain is not unique. Other white, dsRNA-containing strains with this behavior have been isolated from abnormal cankers on European chestnut in northern, central, and southern Italy (*unpublished*).

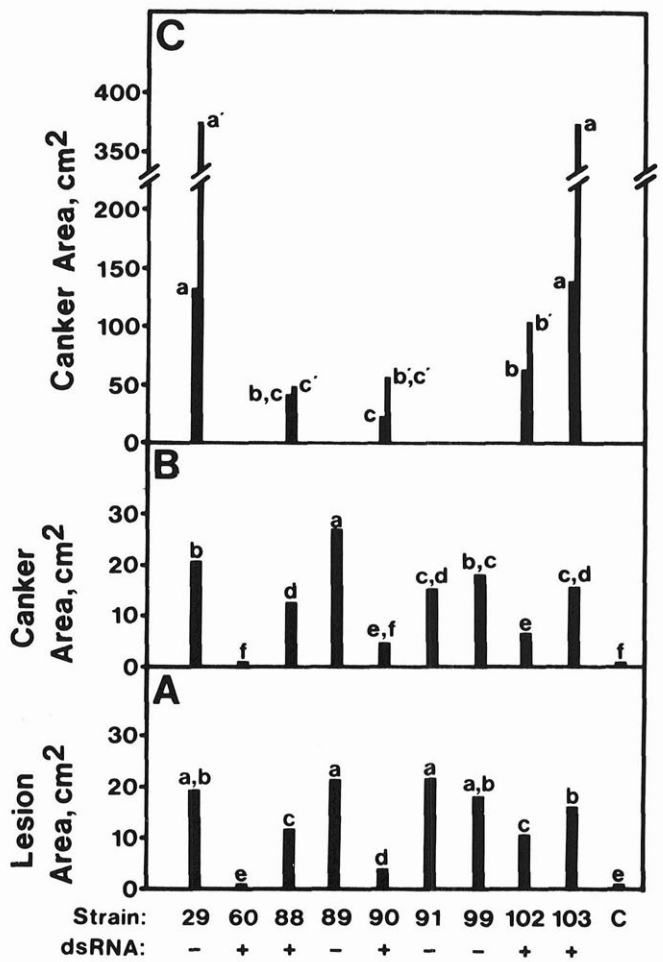

Fig. 6. Growth of American dsRNA-free and ds-RNA containing strains of *Endothia parasitica* in Golden Delicious apples, excised dormant American chestnut stems, and American chestnut sprouts in the field. **A,** Mean lesion area (square centimeters) 15 days after inoculation of Golden Delicious apples. Means are for 10 lesions per strain, five in each of two experiments. **B,** Mean canker area (square centimeters) in dormant American chestnut stems. Means were calculated for 18 cankers per strain, six in each of three experiments. Data were taken from the 33rd, 27th, and 21st days of the first, second, and third experiments, respectively. **C,** Mean canker area (square centimeters) 6 and 13 mo after inoculation into sprouts of American chestnut in the field. Means are for eight cankers per strain. Within a graph, means headed by the same letter are not significantly different according to Duncan's new multiple range test, $P = 0.05$. In graph C, letters without an apostrophe are for 6-mo canker areas and those with an apostrophe are for 13-mo areas. Symbols: C, PDAmb control substituted for inoculum; −, dsRNA-free; and +, dsRNA-containing.

This range of deficiencies suggests that several mechanisms of debilitation may be represented among the strains. Biraghi's (7) and Grente and Berthelay-Sauret's (17) observations that the chestnut host produces periderms which effectively limit spread of the fungus may explain containment of many of these strains, but the bases for the different time courses involved remain to be determined.

No cultural abnormality was consistently associated with reduced pathogenicity. Whiteness in culture was associated with low pathogenicity for most French and French-derived American strains, as reported previously (17,28–30), but not for the Italian or American strains.

All of the dsRNA-containing strains except EP-103 were also deficient in fruiting capacity, especially perithecium formation, compared with the dsRNA-free strains. Of the 10 dsRNA-containing strains pathogenic enough to warrant testing in the field, only three produced perithecia, and two of these strains produced only a few. Also, few of the dsRNA-containing strains fruited as abundantly asexually as the dsRNA-free strains.

Only dsRNA-containing strain EP-103 was indistinguishable from the dsRNA-free strains, except for its minor cultural abnormalities. This indicates that reductions in pathogenicity and fruiting capacity in this fungus are not invariably associated with presence of dsRNA. This is not unexpected in that many fungi are known that contain dsRNA-associated virus-like agents but show no perceptible abnormalities (21).

In summary, no simple tests or indicators were found for quickly and reliably determining if a strain of *E. parasitica* has deficient pathogenicity or fruiting capacity. Of the three methods used, the long-term field tests, with intact chestnut in its natural environment as substrate, was most reliable. Only this test revealed the deficiencies of EP-49, a strain that previously had been described as "virulent" (28). However, the tests with apple fruit and excised dormant American chestnut as substrates were useful for detecting marked deficiencies in pathogenicity.

These findings have several implications. First, the wide range of abnormalities in pathogenicity and fruiting capacity found among the dsRNA-containing strains, all of which were obtained directly or indirectly from naturally occurring abnormal cankers, suggests that a variety of abnormal strains is involved in the natural recovery process. Secondly, if this is so, and if natural recovery of chestnut is to be attributable to hypovirulence in the fungus, then a concept of hypovirulence is needed that accommodates more of this diversity than the concept based upon the French and French-derived American strains. The results of this study suggest that the concept can be broadened, and would gain meaning in relation to natural recovery, if virulence in *E. parasitica* were based on a combined measure of pathogenicity and fruiting capacity. Although this concept of virulence is unconventional, the biology of the fungus and the disease on American and European chestnut provides its justification. Both pathogenicity and fruiting capacity are closely associated with the capacity of the fungus to produce disease (virulence sensu Steen [25]). Both species of chestnut are highly susceptible to *E. parasitica* when the fungus is in its normal state; under favorable environmental conditions, both support rapid growth of the fungus and abundant production of stromata, pycnidia, conidia, perithecia, and ascospores, if both mating types are present. Of the two types of spores produced, ascospores are generally believed to be the primary means by which *E. parasitica* spreads to produce more disease (4,20). Clearly, a strain with a subnormal capacity to colonize bark tissue has a subnormal capacity to cause disease. Also, a strain with a loss or attenuation of its ability to produce spores, especially ascospores, has a major defect in its overall capacity to produce disease. This reproductive disability may play as important a role in the natural recovery process as loss of pathogenicity.

This broadened concept of virulence in *E. parasitica* will be referred to as virulence (sensu lato) and the narrower concept, based on pathogenicity alone, as virulence (sensu stricto). Normal virulence (sensu lato) can be defined as the mean pathogenicity and fruiting capacity of two or more standard, dsRNA-free strains and hypovirulence (sensu lato) as significantly lower virulence, when standards and test strains are compared in the same trees in the field. These definitions are operational and quantitative; determinations of hypovirulence are based on estimates of normal virulence made under the unique conditions of each experiment. The results of this study indicate that such determinations would require at least 6 mo so the perithecial component of virulence (sensu lato) can be evaluated and the subnormal virulence of strains like EP-49 can be detected.

This procedure would identify a general class of hypovirulent strains, but it would provide no information about the cause(s) of their deficiencies. Presumably, the dsRNA found in many of the strains is responsible. The uniformity of the prototrophic dsRNA-free strains, whether obtained as mass mycelial isolates from infected trees or as SCI from dsRNA-containing strains, suggests this, as do results of other investigations (2,9,11). However, other factors may contribute to the diversity of their abnormalities. A recent study (1) which included EP-4, one of the French strains, indicates that part of this strain's deficiency is under nuclear control. Dodds (11) examined a selection of the strains included in this study and concluded that the European and American strains contain different agents. He based this conclusion on differences in the amounts and electrophoretic patterns of dsRNA components from the strains. Also, the variety of dsRNA-containing SCI types obtained from Italian strains EP-49 and 50 suggests that these strains may contain more than one dsRNA-associated agent. Finally, the differences between strains EP-3, 14, 27, and 52 suggest that different genetic backgrounds of the fungus may respond differently to dsRNA from the same source. However, the differences between EP-27 and 43, strains which have the same genetic background and dsRNA from this same source, also suggest that this dsRNA may represent more than one agent.

Clearly a detailed analysis of each hypovirulent strain is needed to determine the cause(s) of its subnormal virulence. Hypovirulence due to cytoplasmic factors, such as dsRNA-associated agents, has been referred to as cytoplasmic hypovirulence (CH) (14–16), or transmissible hypovirulence (TH) (27), and that due to nuclear factors as nuclear hypovirulence (NH) (15). Mixtures of factors in a strain might be resolved by single conidial isolation. This is suggested by the differences found among the SCI of EP-49 and 50 and studies by Grente and Sauret (19) and Bonifacio and Turchetti (8). The relative contributions of cytoplasmic and nuclear factors to a strain's hypovirulence could be determined by transmitting the cytoplasmic agent(s) separately and together, if more than one, into standard strains, determining their effects on the virulence (sensu lato) of the standards, and determining the virulence of the dsRNA-free form of the test strain by comparing it with the standards. This analytic approach, which is described in greater detail elsewhere (16), could facilitate a systematic exploration and classification of the elements that contribute to the diversity now found in *E. parasitica*. Application of this approach to the analysis of hypovirulence (sensu lato) in individual strains will be reported separately.

Although the relations between dsRNA, hypovirulence (sensu lato) in *E. parasitica*, and natural recovery of chestnut may be complex and difficult to ascertain, a better awareness of the diversity that occurs among strains from naturally-occurring abnormal cankers may stimulate wider research and lead to increased understanding of these phenomena.

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