

A Comparison of Five Similar Cytoplasmic Hypovirulent Strains of *Endothia parasitica* from the Southern Appalachian Mountains

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ABSTRACT

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Five culturally similar, debilitated, dsRNA-containing strains, isolated from American chestnut trees growing in the southern Appalachian Mountains and presumed to be *Endothia parasitica*, were studied to establish if they were *E. parasitica* and determine and compare the factors that contribute to their hypovirulence (sensu lato), i.e., subnormal pathogenicity, fruiting capacity, or both. These strains were compared with a strain from western Michigan that has similar abnormalities conferred by dsRNA-associated cytoplasmic hypovirulence (CH) agent H_{M1}. In American chestnut the five southern Appalachian strains were nonpathogenic, like strains containing CH agent H_{M1}, or very weakly pathogenic. Each produced only two types of single-conidial isolates: Isolates like the original strain (usually less than 20%) and isolates with characteristics typical of *E. parasitica*. DsRNA-free single-conidial isolates from all five southern Appalachian strains had cultural characteristics typical of *E. parasitica* and normal levels of virulence (sensu lato) in American chestnut, as determined by comparing them with four standard strains in culture and in American chestnut trees in the field over a 12-mo period. With one minor exception, each of these single-conidial isolates produced perithecia and ascospores typical of *E. parasitica* in the field and

in mating type tests. The five southern Appalachian strains fell into four vegetative compatibility groups. The cytoplasmic agent in each strain was transmitted by hyphal anastomosis directly, or indirectly through intermediary strains, into each of the four standard nuclear genetic backgrounds of *E. parasitica*. Each standard acquired the cultural abnormalities, hypovirulence, and single-conidial isolate segregation pattern of each original strain. Each agent was associated with a consistent pattern of dsRNA components in the original nuclear genetic background and in the two standard backgrounds studied. Four patterns of dsRNA components were found, and none of these was identical to the pattern associated with CH agent H_{M1}. These findings indicate that each of the five southern Appalachian strains is a strain of *E. parasitica* with a nuclear genetic background that confers normal cultural characteristics and a normal level of virulence (sensu lato), and a single dsRNA-associated agent confers its abnormalities. The different patterns of dsRNA components suggest that four CH agents are represented among the five southern Appalachian strains and that these agents differ from CH agent H_{M1} from the Michigan strain. The new agents have been designated H_{NC1}, H_{T1}, H_{T2}, and H_{V1}.

Additional key word: chestnut blight.

Natural recovery of European chestnut, *Castanea sativa* Mill., from chestnut blight in southern Europe and unusual persistence and natural recovery of American chestnut, *C. dentata* Borkh., in the United States have been associated with the presence of abnormal, often swollen, cankers (5-7, 19-21, 26, 28). These cankers usually contain mixtures of abnormal, often weakened, forms and normal forms of the chestnut blight fungus, *Endothia parasitica* (Murr.) P. & H. And. (6, 20, Elliston, *unpublished*). Many of the abnormal forms contain double-stranded RNA (dsRNA) (5, 8, 9, 14, 19, 21, 25), the genetic material of many fungal viruses (23), but the normal forms do not. These observations have suggested that dsRNA-associated, virus-like agents in the abnormal forms are responsible for natural recovery.

Comparison of a collection of European and American dsRNA-free strains and dsRNA-containing strains derived directly or indirectly from abnormal cankers revealed that the dsRNA-free strains had nearly uniform characteristics, but the dsRNA-containing strains displayed diverse abnormalities in cultural characteristics and virulence (sensu lato), i.e., pathogenicity, fruiting capacity, or both. (The broad definitions of virulence and hypovirulence in *E. parasitica* [14] will be used throughout this paper.) Moreover, the array of abnormalities found among the European dsRNA-containing strains differed from that found among the American strains, yet the dsRNA-free strains from both continents were indistinguishable (14). Together, these observations, the observation that representative European and

American strains contain different amounts and patterns of dsRNA components (9) and that dsRNA from representative European and American strains lacked sequence homology (24), indicate that different dsRNA-associated cytoplasmic agents are present in the two populations of the fungus. The diversity of cultural types within each group further suggested that more than one virus-like agent might be represented in each population (14). Therefore, detailed studies of individual abnormal strains clearly are needed, first, to determine that they are *E. parasitica*, second, to determine the bases of their abnormalities and, ultimately, to determine the bases of the observed diversity. The existence of mutant forms with subnormal virulence (3, Elliston, *unpublished*) underscores the need to consider both the contributions of nuclear and cytoplasmic factors in determining the bases of a strain's abnormalities (10).

Strain EP-60, a highly debilitated, dsRNA-containing mass mycelial isolate from recovering American chestnut in western Michigan, was the first strain studied in detail (14-16). The results indicated that EP-60 is a strain of *E. parasitica* with a nuclear genetic background that confers typical cultural characteristics and a normal level of virulence and that it contains two dsRNA-associated cytoplasmic hypovirulence (CH) agents. When present alone in a strain, each agent confers a different level of hypovirulence and different cultural abnormalities. The more debilitating CH agent, designated H_{M1}, is transmitted into few conidia and the less debilitating agent, designated H_{M2}, is transmitted into most conidia produced by infected strains. Each agent had consistent effects in each of 22 European and American nuclear genetic backgrounds of *E. parasitica*. When a strain contained both agents, H_{M1} determined its cultural characteristics and level of virulence.

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Five strains that resembled EP-60 in culture were obtained from cankers on American chestnut trees or sprouts in North Carolina (one strain), Tennessee (three), and Virginia (one). In preliminary studies (18, J. E. Elliston, *unpublished*), each of these southern Appalachian strains appeared to contain one highly debilitating, dsRNA-associated cytoplasmic agent with effects similar to CH agent H_{M1}. The objectives of this study were: to compare the cultural characteristics, pathogenicities, and fruiting capacities of the five southern Appalachian strains, the nuclear genetic background of EP-60 containing CH agent H_{M1}, and the four standard strains; to determine the relative contributions of cytoplasmic and nuclear factors to the abnormalities of the five southern Appalachian strains; to compare the effects that the cytoplasmic agents from these strains have on the cultural characteristics and virulence of the standard strains; to determine how frequently the cytoplasmic agents are transmitted into conidia, and to compare the patterns of dsRNA components associated with the agents in the original and standard nuclear genetic backgrounds. A preliminary report of this work has been given (17).

MATERIALS AND METHODS

The following strains, known or presumed to be *E. parasitica*, were used most extensively: a) standard strains: I, EP-155, a dsRNA-free mass mycelial isolate from CT; II, EP-408, a dsRNA-free mass mycelial isolate from Mt. Senario, Tuscany, Italy; III, EP-421, a dsRNA-free single-conidial isolate from a dsRNA-containing mass mycelial isolate from Mt. Amiata, Tuscany, Italy;

and IV, EP-523, a dsRNA-free single-conidial isolate from EP-60, a dsRNA-containing mass mycelial isolate from Rockford, MI (15); b) EP-544, a dsRNA-free single-conidial isolate with the nuclear genetic background of EP-60 and containing dsRNA-associated CH agent H_{M1}, after this agent had traversed a 10-step transmission cycle (16); and c) the five southern Appalachian strains: EP-1103, a mass mycelial isolate from an American chestnut sprout in Iredell County, NC (provided by Bruce Nash and William Stambaugh, Duke University, Durham); EP-232, 234, and 237, mass mycelial isolates from abnormal cankers located 24, 27, and 31 ft (7.3, 8.2, and 9.5 m), respectively, above ground level on an American chestnut tree 25 cm in diameter at a height 1.4 m above ground level, growing near Belair, TN (provided by David McCarroll and Eyvind Thor, University of Tennessee, Knoxville); and EP-700, a mass mycelial isolate from a large superficial canker on an American chestnut sprout near Natural Bridge, VA (provided by E. G. Kuhlman, USDA Forest Service, Research Triangle Park, NC). For convenience, the single-conidial isolate from EP-60 containing CH agent H_{M1} and the five southern Appalachian strains listed above will be referred to as the original strains and given the designations, O₁, O₂, O₃, O₄, O₅, and O₆, respectively.

Cultures were maintained and grown for inoculum and determination of cultural characteristics on Difco potato-dextrose agar amended with 100 mg of L-methionine and 0.1 mg of biotin per liter (PDAMB) under standard conditions (12).

Single-conidial isolation experiments were made as described (21). Single-conidial isolates from an original strain were referred to as first-generation single-conidial isolates, and those from a first-generation single-conidial isolate were referred to as second-generation single-conidial isolates.

Cytoplasmic agents were transmitted from the original to the standard strains by hyphal anastomosis in bark of excised American chestnut or in culture (16), using additional nuclear genetic backgrounds of *E. parasitica* as needed to circumvent barriers to transmission posed by vegetative compatibility (v-c) (2,4,5,22).

Patterns of dsRNA components were determined for the original strains and the infected forms of standard strains II and IV, the latter representing typical nuclear genetic backgrounds from the southern European and North American populations of the fungus, respectively. Composite samples also were tested to determine if the bands from all three strains in each set electrophoresed together. Composite samples were prepared by mixing equal quantities of the mycelium of each strain before extraction. Strains were tested for dsRNA as described (16), except up to 7.5 g fresh weight of each strain was extracted. Extracts representing up to 3.5 g fresh weight of mycelium were electrophoresed. Gels were stained with propidium iodide and photographed. Susceptibility of the propidium iodide-stained bands to digestion by ribonuclease in high and low salt solution was determined by the method of Dodds (9).

Virulence of strains in American chestnut was estimated as described (16), using the four standard strains to provide estimates of normal virulence, except that two experimental designs were used. In the first design, standard strains were assigned to one side of each of four trees according to a 4 × 4 Latin square design to permit any effect of position on the trunk to be detected, and test strains were assigned to the opposite side according to a randomized complete block design. In the second design, a superimposed Latin square, the four standard strains were assigned to one side of each of four trees as described above, and the set of subcultures of each standard, containing CH agent H_{M1} and the cytoplasmic agents from the five test strains, was assigned to the other side of the trunk in the region opposite the corresponding standard strain. The subcultures in each set were arranged in random order. All trees were at least 7.5 cm in diameter at a height of 1.4 m above ground level, and only regions of the trunk with smooth bark were inoculated. All inoculations were made in late May or early June.

Tests of mating type were made as described (15), using EP-42 and 106 as *A* and EP-67 and 107 as *a* mating type testers, respectively.

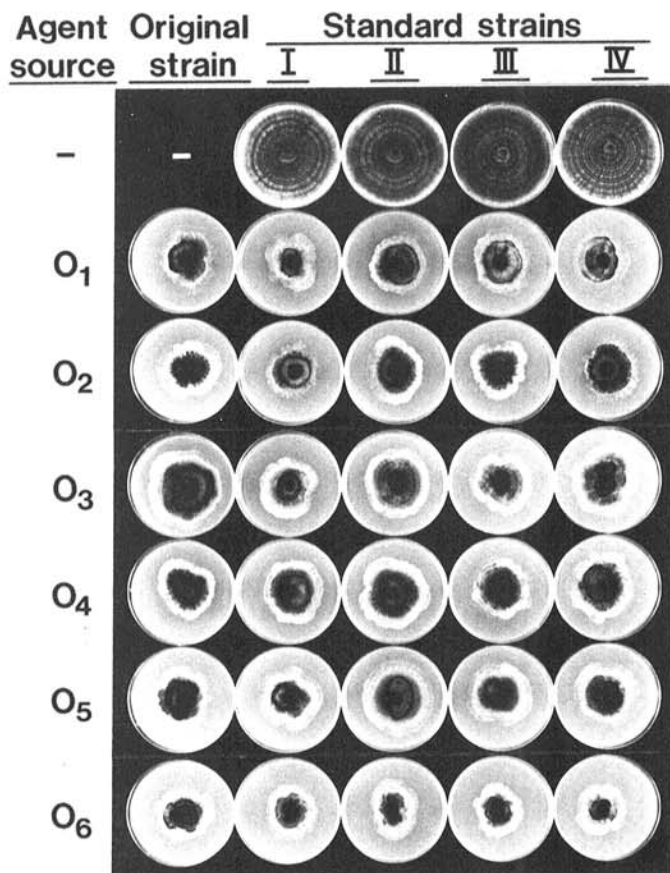


Fig. 1. Seven-day-old cultures of the original strains, O₁ (EP-544) from western Michigan, O₂ (EP-1103) from North Carolina, O₃, O₄, and O₅ (EP-232, 234, and 237, respectively) from Tennessee, and O₆ (EP-700) from Virginia; standard strains, I (EP-155), II (EP-408), III (EP-421), and IV (EP-523); and the standard strains containing the debilitating cytoplasmic agent from each original strain, grown on PDA plus methionine and biotin at 20 ± 2 C with a 16-hr photoperiod.

RESULTS

Typical 7-day-old colonies of the six original strains are shown in Figure 1. All six had abnormal features resembling those described for EP-60 (15); most notably, slow, asymmetric growth, leading mycelium varying from sparse to densely packed, little aerial mycelium and thalli that varied widely in shape and in thickness and color from one region to another.

Of the six original strains, only O₁, O₅, and O₆ were nonvirulent. Strain O₃ was most virulent, but all were markedly hypovirulent compared with the standard strains, and none produced perithecia within the 12-mo duration of the test. During the same period, the standard strains produced large cankers with abundant stromata and perithecia (Fig. 2A and B).

Each original strain yielded two types of single-conidial isolates through two successive generations: one type like the parent strain and one with characteristics typical of *E. parasitica* in its normal state (14). In all cases, only a small proportion of the single-

conidial isolates were like the parent strain (Table 1).

Representative normal single-conidial isolates from the original strains, designated N₁ through N₆, had normal levels of virulence, whether the determination was made 2 or 12 mo after inoculation (Fig. 2C and D, respectively). Each of these isolates produced large cankers with abundant stromata, perithecia, and two-celled ascospores typical of *E. parasitica*, except that the perithecia produced by the normal single-conidial isolate from O₆ had white instead of black necks. The mating types of the normal single-conidial isolates from O₂, O₃, O₅, and O₆ were *A*, and the isolate from O₄ was *a*. The normal single-conidial isolates from O₃, O₄, O₅, and O₆ were assigned to v-c groups 44, 24, 44, and 49, respectively. The v-c designation for the normal isolate from O₂ was not determined.

The cytoplasmic agents were transmitted from the original

TABLE 1. Percentages of single-conidial isolates (SCI) with the abnormal cultural characteristics of the original strains, produced by the original strains and the infected standard strains

Source of CH ^b agent	Percent SCI like original strain (total SCI)				
	Original strain	Standard strains ^a			
		I	II	III	IV
O ₁ ^c	17 (511)	5 (123)	4 (99)	0 (180) ^d	22 (417)
O ₂	2 (162)	15 (111)	10 (114)	7 (100)	9 (115)
O ₃	18 (315)	17 (122)	11 (152)	20 (101)	4 (109)
O ₄	7 (411)	3 (96)	1 (86)	3 (103)	9 (223)
O ₅	10 (542)	20 (129)	11 (122)	4 (106)	2 (110)
O ₆	2 (224)	8 (258)	5 (96)	2 (193)	3 (222)

^a I = EP-155; II = EP-408; III = EP-421; IV = EP-523.

^b Cytoplasmic hypovirulence.

^c O₁ = EP-544 from Michigan; O₂ = EP-103 from North Carolina; O₃, O₄, and O₅ = EP-232, 234, and EP-237, respectively, from Tennessee; and O₆ = EP-700 from Virginia.

^d Drops of spore suspensions containing 25 or more conidia produced colonies like the original strain.

TABLE 2. Pathways used to transmit the cytoplasmic agents from the original to the standard nuclear genetic backgrounds of *Endothia parasitica*

Standard (v-c group ^a)	Cytoplasmic hypovirulence agent ^b	Pathway (sequence of v-c groups)
I (40)	H _{M1}	9-8-40
II (12)	H _{M1}	9-12
III (11)	H _{M1}	9-11
IV (9)	H _{M1}	9-8-9
I (40)	H _{NC1}	x ^c -40
II (12)	H _{NC1}	x-40-12
III (11)	H _{NC1}	x-40-11
IV (9)	H _{NC1}	x-9
I (40)	H _{T1}	44-8-40
II (12)	H _{T1}	44-8-9-11-12
III (11)	H _{T1}	44-8-9-11
IV (9)	H _{T1}	44-8-9
I (40)	H _{T2}	24-8-40
II (12)	H _{T2}	24-8-40-12
III (11)	H _{T2}	24-8-9-11
IV (9)	H _{T2}	24-8-9
I (40)	H _{T3}	44-8-40
II (12)	H _{T3}	44-8-9-12
III (11)	H _{T3}	44-8-40-11
IV (9)	H _{T3}	44-8-9
I (40)	H _{V1}	49-40
II (12)	H _{V1}	49-40-12
III (11)	H _{V1}	49-11
IV (9)	H _{V1}	49-9

^a Vegetative-compatibility group.

^b The designation H_{M1} was used for the cytoplasmic hypovirulence (CH) agent from *E. parasitica* strain EP-544, and designations H_{NC1}, H_{T1}, H_{T2}, H_{T3}, and H_{V1} were tentatively used for the CH agents from strains EP-1103, 232, 234, 237, and 700, respectively.

^c The v-c group of this strain was not determined.

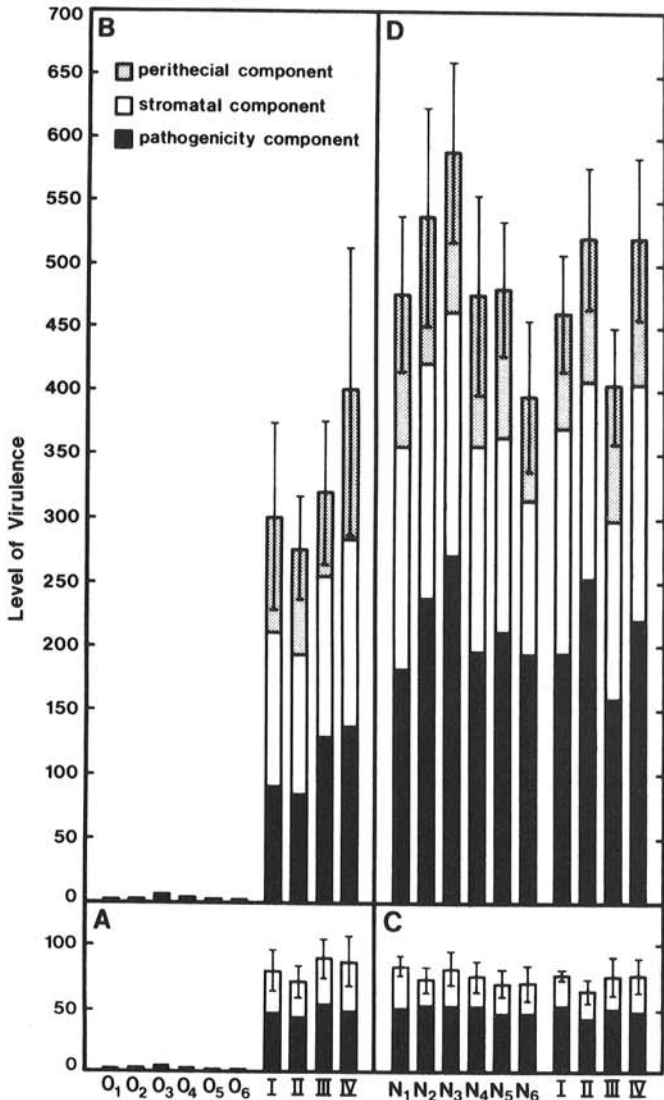


Fig. 2. Estimated virulences of the six original strains, O₁ (EP-544) from western Michigan, O₂ (EP-1103) from North Carolina, O₃, O₄, and O₅ (EP-232, 234, and 237, respectively) from Tennessee, and O₆ (EP-700) 12 from Virginia; and the four standard strains, I (EP-155), II (EP-408), III (EP-421), and IV (EP-523); A, 2 mo and B, 12 mo after inoculation of four American chestnut trees; and estimated virulences of one normal-appearing, dsRNA-free single-conidial isolate, N₁ through N₆, from original strains O₁ through O₆, respectively; and the four standard strains, I through IV; C, 2 mo and D, 12 mo after inoculation of four American chestnut trees. Virulence was calculated as the sum of the mean canker area in square centimeters, the mean number of stromata divided by 10, and the mean number of stromata containing perithecial beaks divided by 10. Bars represent standard errors.

strains to the standard strains by the pathways shown in Table 2. Typical 7-day-old colonies of standard strains I-IV, with and without the CH agents from the six original strains, are shown in Figure 1. In single-conidial isolation experiments, each of the 24 infected standard strains yielded two types of single-conidial isolates in approximately the same proportions as the original strains (Table 1). The standard strains containing these agents had negligible virulence compared with the same strains in their uninfected states (Fig. 3A and B). However, the cytoplasmic agent from the most virulent of the original strains, O₃, also allowed the greatest virulence in the standard strains. The agents from original strains O₂, O₃, O₄, O₅, and O₆ were tentatively designated H_{NC1}, H_{T1}, H_{T2}, H_{T3}, and H_{V1}, respectively.

The original strains and the standard strains containing the cytoplasmic agents from the original strains all contained dsRNA (Fig. 4). No dsRNA was detected in the normal single-conidial isolates from the original strains (not shown). The pattern of dsRNA components associated with each agent appeared to be the same in the original and both standard strains. Only the patterns associated with original strains O₄ and O₅, strains that came from the same tree, appeared to be identical. (The apparent discrepancy between this statement and the dsRNA patterns for O₄ and O₅

shown in Figures 4 and 5 probably are due to differences in concentration. This difference was not evident in other experiments in which the intensities of the bands associated with these agents were high.)

The differences in patterns of dsRNA components were explored further by mixing equal quantities of extracts in all possible pairs, subjecting the mixtures and equal quantities of unmixed extracts to electrophoresis, and comparing the patterns of bands. This procedure confirmed that only the patterns for the agents from O₄ and O₅ were identical, and revealed that the heaviest (slowest-migrating) component associated with the agent from O₃ is heavier than the heaviest component from O₄ and O₅, and that both of these components are heavier than the heaviest components associated with the other three agents. This is shown diagrammatically in Figure 5.

DISCUSSION

The results of this study indicate that each of the five abnormal southern Appalachian strains is, like EP-60 from Michigan (15), a form of *E. parasitica* with a nuclear genetic background that confers normal cultural characteristics and a normal level of

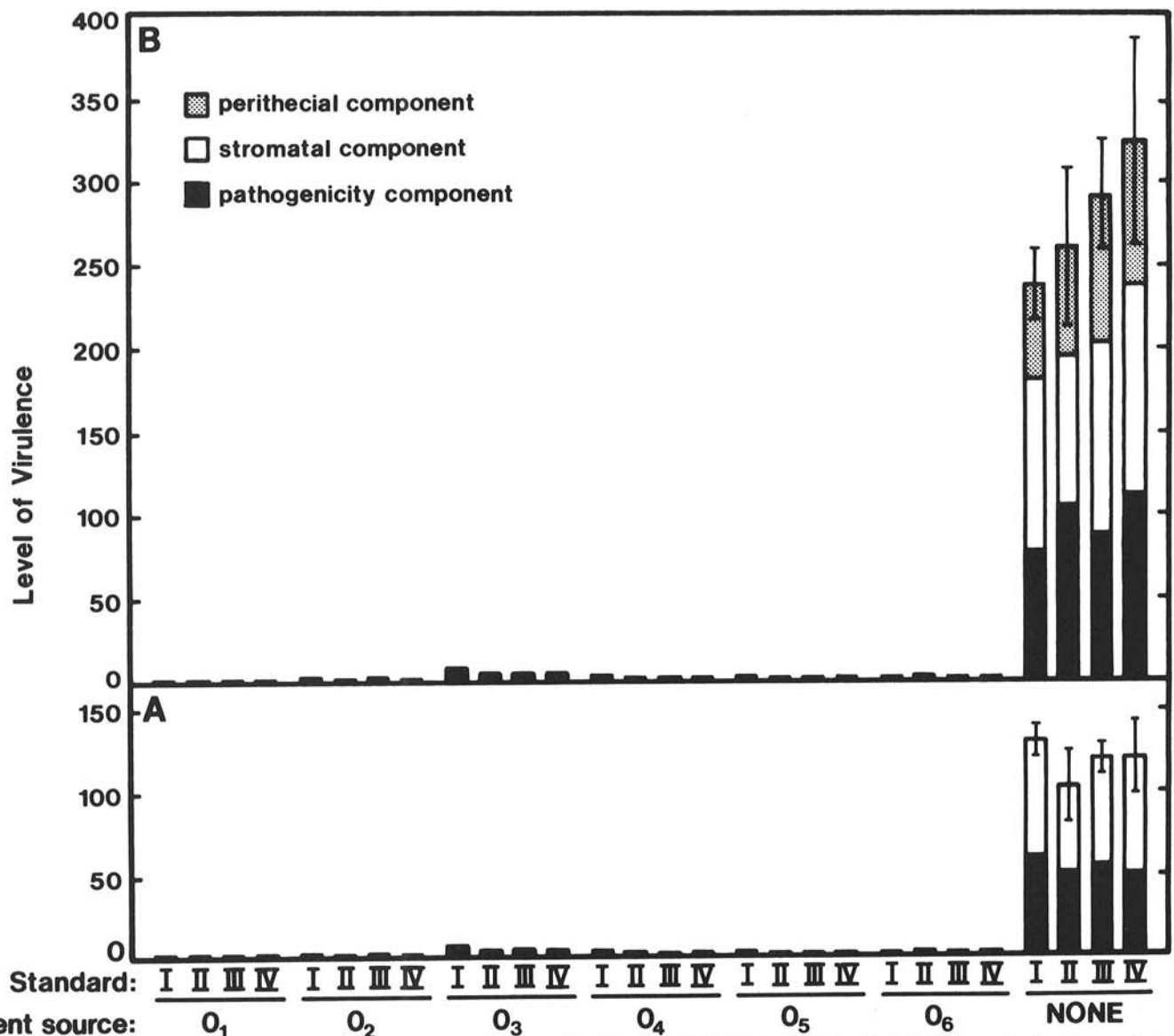


Fig. 3. Estimated virulences of standard strains, I (EP-155), II (EP-408), III (EP-421), and IV (EP-523) containing the debilitating cytoplasmic agent from each original strain, O₁ (EP-544) from western Michigan, O₂ (EP-1103) from North Carolina, O₃, O₄, and O₅ (EP-232, 234, and 237, respectively) from Tennessee, and O₆ (EP-700) from Virginia, and in their uninfected states (none); A, 2 mo and B, 12 mo after inoculation into four American chestnut trees. Virulence was calculated as the sum of the mean canker area in square centimeters, the mean number of stromata divided by 10, and the mean number of stromata containing perithecial beaks divided by 10. Bars represent standard errors.

virulence. However, unlike EP-60, each of these strains contains only one debilitating dsRNA-associated cytoplasmic agent that confers abnormal cultural characteristics and low virulence.

The conclusion that these strains are *E. parasitica* is based on the characteristics of dsRNA-free single-conidial isolates obtained from them. The cultural characteristics of these isolates and their levels of virulence in American chestnut were not significantly different from those of the four standard strains, which are representative dsRNA-free strains taken from the North American

and southern European populations of the fungus (11). More importantly, in the field and when mated with mating type testers of *E. parasitica* in the laboratory, each of these isolates produced ascospores typical of the species. Only the strain from Virginia was atypical: It produced perithecia with white instead of the usual black necks.

The conclusion that each strain contained one dsRNA-associated cytoplasmic agent is based largely on the repeating, dichotomous patterns of single-conidial isolate segregation. Although a dichotomous segregation could occur among first-generation single-conidial isolates if the parent strain were heterokaryotic, a dichotomous segregation through two generations of single-conidial isolates can only be explained by the presence of one cytoplasmic agent. The transmission of each agent through up to three other nuclear genetic backgrounds and into four standard strains, the dichotomous single-conidial isolate segregations of single-conidial isolates from the infected standards, and the consistent patterns of dsRNA components in each set of

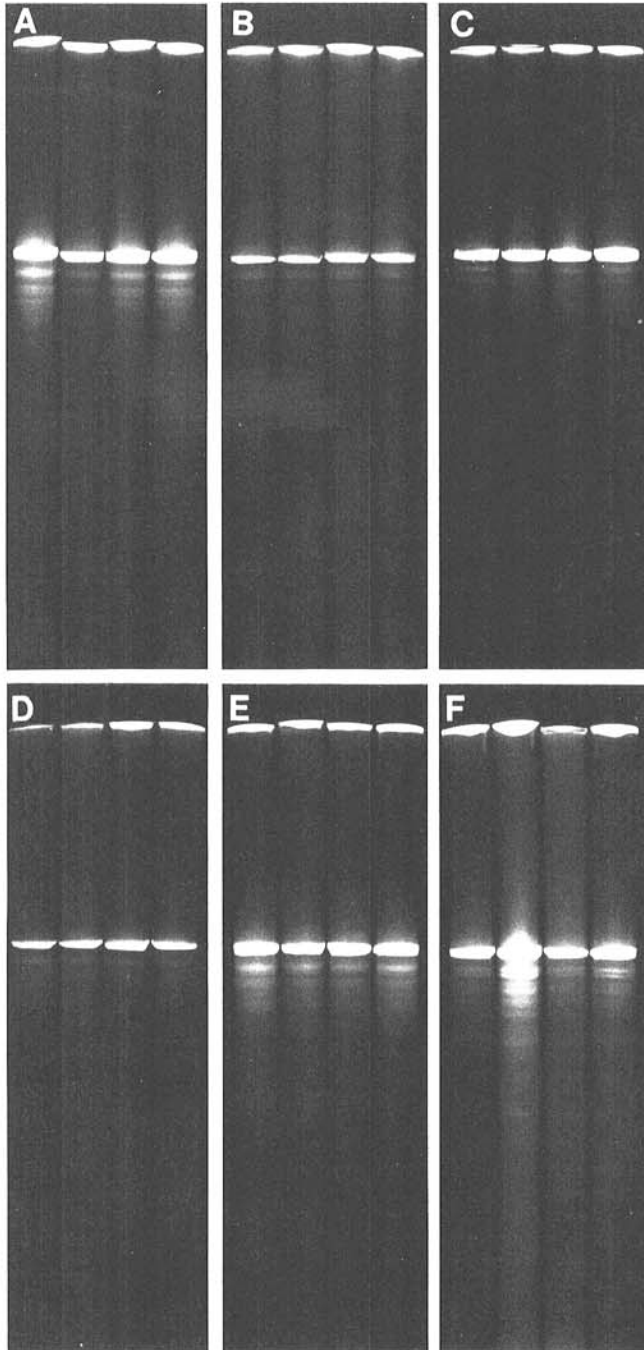


Fig. 4. Sets of polyacrylamide gels showing patterns of dsRNA components associated with the cytoplasmic agents from original strains **A**, O_1 (EP-544) from western Michigan; **B**, O_2 (EP-1103) from North Carolina; **C-E**, O_3 , O_4 , and O_5 (EP-232, 234, and 237, respectively) from Tennessee; and **F**, O_6 (EP-700) from Virginia. Each set includes, from left to right, gels for the original strain, standard strain II (EP-408), standard strain IV (EP-523), and a composite sample. Composite samples consisted of equal quantities of mycelium of the three strains in each set. Extracts equivalent to 3.5 g fresh weight were electrophoresed on 5% gels 80 mm long for 12 hr at 6 mA per gel. Gels were stained with propidium iodide and photographed while being exposed to ultraviolet light.

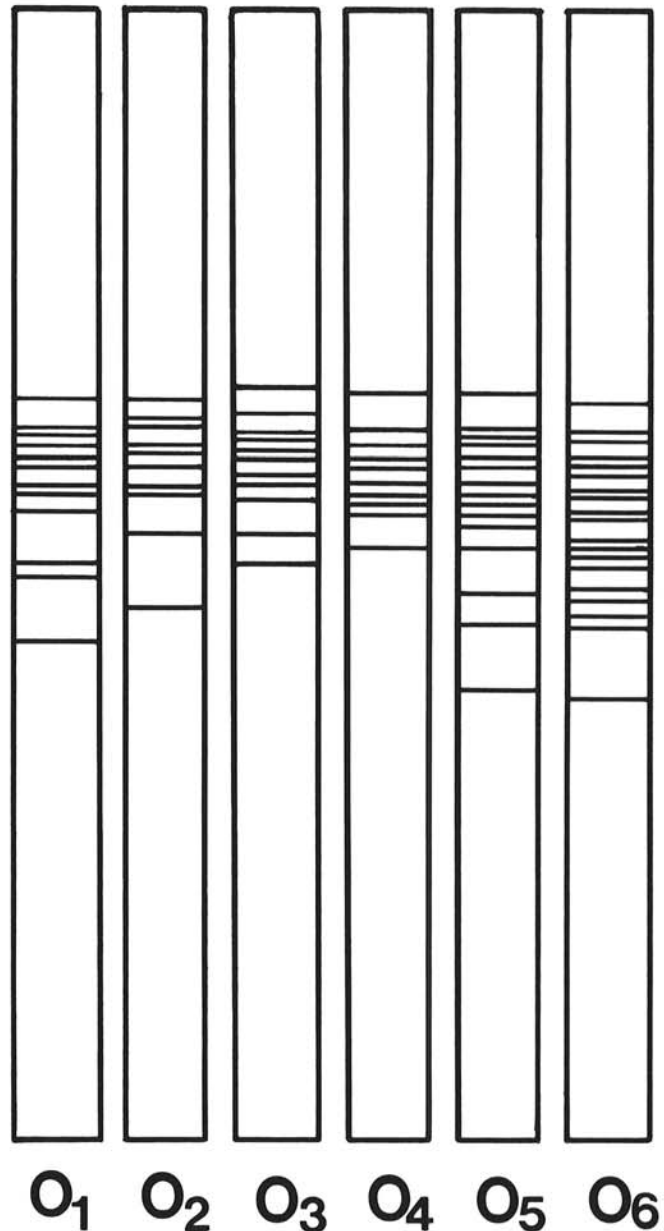


Fig. 5. Diagrammatic representation of patterns of dsRNA components associated with the debilitating cytoplasmic agents in original strains O_1 (EP-544) from western Michigan, O_2 (EP-1103) from North Carolina, O_3 , O_4 , and O_5 (EP-232, 234, and 237, respectively) from Tennessee, and O_6 (EP-700) from Virginia. Relative positions of bands were determined by mixing and electrophoresing extracts in all possible pairs.

original and infected standard strains also support this conclusion.

Both in this study and in the study of strain EP-60 (16), the sets of dsRNA components and cultural abnormalities associated with each cytoplasmic agent appeared to be stable and consistent in several European and North American nuclear genetic backgrounds of the fungus and following transmission between up to five nuclear genetic backgrounds. Unstable patterns of dsRNA components, cultural characteristics, or both, have been reported with other dsRNA-containing strains in earlier studies (1,4,27). The stability encountered during the course of this study, which spanned more than 6 yr, may be a consequence of working with single infections, or it may be peculiar to the cytoplasmic agents involved. However, it is possible that the strains used in earlier studies of stability contain more than one agent, as strain EP-60 does (16), and these agents were segregating when mycelium was transferred or dsRNA was transmitted between strains. Further studies of those strains may reveal the basis for these differences.

The agents from the five southern Appalachian strains and agent H_{M1} from EP-60 affected similarly the cultural characteristics and virulences of the source strains and the four standard strains and were transmitted into conidia of all these strains with low frequencies. These similarities suggest that the agents may be closely related. However, the patterns of dsRNA components associated with four of the five agents from these strains differed from one another and from the pattern associated with agent H_{M1} from the Michigan strain, EP-60. Thus, it appears that five somewhat different dsRNA-associated CH agents are represented among the six compared in this study; the agents designated H_{T2} and H_{T3} appear to be identical. RNA hybridization studies, such as those reported by L'Hostis et al (24), are needed to further explore the relatedness of these agents and to aid the search for their sources (12,13).

Whether or not the cytoplasmic agents present in the six original strains are closely related, their remarkably similar effects on the biological properties of *E. parasitica* put the infected strains into a natural form-class within the overall population of abnormal strains. The effects these agents have on colony morphogenesis are fascinating and might serve as the basis of an illuminating study in fungal pathology. At the outset of colony development, a strain containing one of these agents typically grows from the inoculum plug rather vigorously, but more rapidly from the side that was nearest to the leading edge of the inoculum colony. Then much or all of the leading mycelium stops growing, thickens, and eventually a few hyphae emerge from the leading edge of the thickened initial colony, but often not synchronously or uniformly. This process is repeated several times, but seldom in synchrony about the colony margin, so a highly asymmetric colony usually develops (Fig. 1). The physiological and molecular bases for this pattern of development are unknown but may be related in some way to cycles of dsRNA replication and accumulation that are not coordinated within the colony. Strains of *E. parasitica* infected with a number of other CH agents from southern Europe and North America develop symmetrically (11), suggesting that these agents act continuously during colony development. These contrasting types of development suggest that different types of CH agents may have quite different mechanisms of pathogenesis in the fungus.

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