Further Evidence for Two Cytoplasmic Hypovirulence Agents in a Strain of *Endothia parasitica* from Western Michigan

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ABSTRACT

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Correlative evidence supported the hypothesis that two cytoplasmic agents confer the abnormalities of EP-60, the first highly debilitated, dsRNA-containing strain of *Endothia parasitica* found in North America. The nuclear genetic background of EP-60 conferred normal cultural characteristics and normal virulence (sensu lato) in American chestnut, as determined by comparing it with three standard strains. The putative agents were transmitted both separately and together, by hyphal anastomosis and through conidia, in a cycle from the nuclear genetic background of EP-60 to the background of EP-6, an unrelated, marked (methionine-requiring) strain, and back to the nuclear genetic background of EP-60, approximately fulfilling Koch's postulates for the agents separately and together. Four infection states, the number predicted for two independent cytoplasmic agents, occurred as three cultural types. Each infection state was associated with a different combination of cultural characteristics, pathogenicity, SCI segregation pattern, and pattern of dsRNA components. Each agent conferred consistent cultural abnormalities on 20 other North American and European nuclear genetic backgrounds of *E. parasitica*, including the three standard backgrounds and seven that previously contained other dsRNA-associated agents. Each agent also similarly affected the pathogenicities and virulences of representative North American and European nuclear genetic backgrounds, including the standards. The infection state of EP-60 was established in the nuclear genetic backgrounds of EP-60, EP-6, and the standards by hyphal anastomosis between corresponding singly infected forms. A different, but consistent, pattern of dsRNA components was associated with each agent in the nuclear genetic backgrounds of EP-60 and the three standards. These patterns appeared to be combined when both agents were present. The more and less debilitating agents were transmitted via about 10 and 90% of conidia, respectively. A mycelial slurry containing isolates with both agents had marked curative effects when introduced into naturally occurring cankers. These observations confirm that EP-60 has a nuclear genetic background that is typical of *E. parasitica* and demonstrate that it contains two independent cytoplasmic agents which, when present alone in many nuclear genetic backgrounds of *E. parasitica*, confer different degrees of hypovirulence and, when together, have marked curative effects on developing cankers. The more and less debilitating cytoplasmic hypovirulence agents were designated H_{M1} and H_{M2}, respectively.

Additional key words: *Castanea dentata*, chestnut blight.

Weakened, dsRNA-containing strains of the chestnut blight fungus, *Endothia parasitica* (Murr.) P. and H. And., have been associated with natural recovery and unusual persistence of American chestnut, *Castanea dentata*, in North America (9,10,20,27,29,39) and European chestnut, *C. sativa*, in southern Europe (6,10,20). Presence of dsRNA, the genetic material of many fungal viruses (32), suggests that viruslike pathogens of the fungus are involved. Dodds (13) suggested that American and European strains contain different viruslike agents based on differences in the amounts and patterns of dsRNA components. The diverse abnormalities of dsRNA-containing strains from both regions further suggest that a variety of dsRNA-associated agents may be involved (15,20). However, little is known about the agents or about the infection state (i.e., the number of agents or factors present) of any dsRNA-containing strain. Because the dsRNA-associated agents are cytoplasmic (7,38) and reduce the ability of the fungus to cause disease, they have been called cytoplasmic hypovirulence (CH) agents; the strains containing them, CH strains; and the phenomenon, CH (16,17). Synonyms include exclusive hypovirulence (25), contagious hypovirulence (24), infectious hypovirulence (30), and transmissible hypovirulence (38).

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Nuclear factors or combinations of nuclear and cytoplasmic factors also could debilitate some strains. Reduced virulence attributable to nuclear factors has been called nuclear hypovirulence (17) or nontransmissible hypovirulence (37).

Results of a previous study (20) indicated the need for detailed study of individual strains to determine what factors contribute to their debilitation. That study also showed the need for broad operational definitions of virulence and hypovirulence in *E. parasitica* that accommodate cytoplasmic, nuclear, and other possible types of hypovirulence and maintain a conceptual link between hypovirulence and the natural recovery process. The scheme outlined and operational definitions offered were applied with further elaboration in this study of strain EP-60 from western Michigan, the first nonpathogenic, dsRNA-containing strain found in North America (22). In the first part of the study (21), the infection state of EP-60 was investigated by determining patterns of single-conidial isolate (SCI) segregation for the parent strain and representative SCI. The results suggested that EP-60 has a nuclear genetic background typical of *E. parasitica* and that it contains two CH agents, one more debilitating than the other. Of the four infection states predicted for SCI derived from a strain containing two agents, only three were tentatively identified: type A SCI, with both agents; type B, with the less-debilitating agent; and type C, with neither agent having characteristics typical of *E. parasitica*.

This part of the study had the following objectives: (i) to seek the fourth infection state; (ii) to determine if Koch's postulates can be approximately fulfilled for the putative agents separately and together by transmitting them, by hyphal anastomosis and through conidia, in a cycle from the nuclear genetic background of EP-60 to

an unrelated methionine-requiring auxotroph and back to the nuclear genetic background of EP-60 and comparing their effects in each background; (iii) to determine the effects of each agent when introduced into a wide variety of European and North American nuclear genetic backgrounds of *E. parasitica*, including backgrounds which previously contained other dsRNA-associated agents; (iv) to determine if the infection state of EP-60 can be reestablished from its component parts in the nuclear genetic background of EP-60 and in representative American and European backgrounds; (v) to determine the patterns of dsRNA components associated with the agents when alone and together in the nuclear genetic background of EP-60 and in three standard backgrounds; (vi) to determine the agents' effects alone and together on the virulence of standard backgrounds; (vii) to determine the level of virulence conferred by the nuclear genetic background of EP-60 compared with standard backgrounds; and (viii) to determine if the agents together have curative effects when introduced into typical, naturally occurring blight cankers on American chestnut. Preliminary reports have been given (14,19,22).

TABLE 1. Pertinent information for strains of *Endothia parasitica* used in a study of hypovirulence-producing agents

Strain	ATCC no.	Isolate form	Cultural type[a]	vc group[b]	Geographic origin
EP-6[c]	22508	SCI[d]	C	8	Connecticut
EP-15	-	SCI	C	20	Connecticut
EP-29	38754	MAI[e]	C	16	Connecticut
EP-30	-	MMI[f]	C	24	Pennsylvania
EP-38	-	MMI	C	25	Tennessee
EP-39	-	MMI	C	9	West Virginia
EP-40	-	MMI	C	26	West Virginia
EP-42	38751	MMI	C	5	Connecticut
EP-46	-	MMI	C	11	Tuscany, Italy
EP-55	-	MMI	C	27	West Virginia
EP-58	-	MMI	C	22	Connecticut
EP-59	-	MAI	C	9	Connecticut
EP-60	38765	MMI	A	9	Michigan
EP-62	-	SCI[g]	C	12	Tuscany, Italy
EP-65	-	SCI[g]	C	11	Tuscany, Italy
RP-67	38753	SCI[g]	C	10	Tuscany, Italy
EP-74	-	SAI[h]	C	18	Connecticut
EP-89	-	MMI	C	14	Michigan
EP-106	38746	SAI	C	1	Connecticut
EP-107	38747	SAI	C	2	Connecticut
EP-108	38748	SAI	C	3	Connecticut
EP-110	-	SAI	C	5	Connecticut
EP-111	-	SAI	C	6	Connecticut
EP-144	-	MMI	C	30	West Virginia
EP-148	-	SAI	C	33	Connecticut
EP-149	-	SAI	C	34	Connecticut
EP-150	-	SAI	C	35	Connecticut
EP-154	-	SAI	C	39	Connecticut
EP-155	38755	MMI	C	40	Connecticut
EP-161	-	MMI	C	46	Connecticut
EP-165	-	MMI	C	50	West Virginia
EP-169	-	MMI	C	15	West Virginia
EP-305	-	MMI	C	40	Connecticut
EP-366	-	SAI	C	39	Connecticut
EP-393[c]	38984	SAI	C'	5	Connecticut
EP-408	-	MMI	C	12	Tuscany, Italy
EP-409	-	MMI	C	40	Tuscany, Italy
EP-421	-	SCI[g]	C	11	Tuscany, Italy
EP-427	-	MMI	C	40	Piedmont, Italy
EP-432	-	MMI	C	30	Campania, Italy
EP-462	-	MMI	C	30	Campania, Italy
EP-502	-	MMI	C	10	Connecticut
EP-505	-	SCI[g]	C	40	Piedmont, Italy
EP-516[c]	-	SCI	C	8	Connecticut
EP-518	-	SCI[g]	A	9	Michigan
EP-523	-	SCI[g]	C	9	Michigan
EP-524	-	SCI[g]	B	9	Michigan
EP-589	-	SCI[g]	C	24	Tennessee
EP-709	-	SCI[g]	C	49	Virginia

[a] Four cultural types were distinguished: A, like EP-60: colonies small, with irregular shapes, thin advancing mycelium, little aerial mycelium, and usually with a thickened, dark brown disk of mycelium at the center; B, radially striated, symmetrical, yellowish to orangish-brown colonies of intermediate size, with moderate aerial mycelium, and pycnidia arranged in a continuous convoluted mass near the colony center and in concentric rings nearer the margin; C, typical of *Endothia parasitica*: large, radially striated, symmetrical orange colonies with abundant aerial mycelium, and pycnidia arranged in concentric rings; C', a mutant with characteristics like C except colony color is cream.
[b] Determined by S. L. Anagnostakis.
[c] Methionine-requiring auxotroph.
[d] Single-conidial isolate (SCI).
[e] Mass-ascospore isolate.
[f] Mass-mycelial isolate.
[g] SCI of a dsRNA-containing strain.
[h] Single-ascospore isolate.

MATERIALS AND METHODS

Diverse strains of *E. parasitica* were used: these included EP-60, selected SCI from EP-60 with cultural types and infection states A, B, and C from the previous study (21); orange and cream-colored methionine-requiring mutants; and typical strains from six states in the United States and three regions of Italy (Table 1). These represented 29 vegetative compatibility (v-c) groups and included three American and five Italian type C SCI from naturally occurring, dsRNA-containing strains. 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 (20). Single-conidial isolation experiments were made as described (29). The following terms were adopted to describe the results: first generation SCI = SCI from the first strain in a sequence of single-conidial isolation experiments; second generation SCI = SCI from a first generation SCI; a nonsegregating strain = one that yielded only one cultural type of SCI; a segregating strain = one that yielded two or more cultural types of SCI; and the SCI segregation pattern of a strain = the set of SCI cultural types it produced. In later experiments, to confirm that type C SCI from a given strain were nonsegregating without conducting laborious single-conidial isolation experiments, five spore masses from each of six SCI were transferred to separate plates of PDAmb and incubated with spore masses from appropriate controls. This simpler procedure was adopted because conidia in a spore mass, being of like genotype, should germinate and anastomose freely, allowing any debilitating cytoplasmic agent(s) present in even a few conidia to multiply, spread throughout the developing thallus, and be detected. Also, because a spore mass contains vastly more conidia than are sampled in a typical single-conidial isolation experiment, the spore mass procedure is more sensitive.

Pathogenicity of *E. parasitica*, defined as the level of its capacity to grow in and kill chestnut bark tissue, was determined in intact trees as described (20), except four to eight trees were used in each test and all strains in a test were inoculated into each tree. Canker areas were determined 2 or 3 mo after inoculation.

Cytoplasmic agents were transmitted by hyphal anastomosis from donor to recipient strains in bark of excised dormant American chestnut stems (20) or in culture. All transmissions between the nuclear genetic backgrounds of EP-60 and EP-6 (an orange, methionine-requiring auxotroph) were made in chestnut bark as follows: three slightly overlapping 7-mm-diameter holes were made at each inoculation site to the depth of the cambium and parallel with the long axis of the stem. Plugs of donor inoculum were inserted into the first and third holes, plugs of recipient inoculum were placed in the center hole, then the sites were covered with masking tape to retard drying. Inoculated stems were incubated at 20 ± 2 C for 3–6 wk (20). Isolations were made from six to eight points equally spaced around the margin of each canker. To detect transmission, isolates were grown on PDAmb under standard conditions to determine cultural characteristics and plated with appropriate controls on minimal medium (36),

prepared with Noble agar ± 0.1 g L-methionine per liter, to determine methionine-requirement.

In culture, donors and recipients were paired on PDAmb (40 ml) in 150 × 15-mm petri dishes. Three approximately 3 × 5-mm blocks each of donor and recipient inoculum, cut with a flamed scalpel from the leading edges of actively growing colonies, were placed 8 mm apart on opposite sides of a line drawn down the center of each dish. Blocks were aligned about 4 mm apart across the line and oriented with mycelium facing down and leading mycelium directed toward the line. Plates inoculated similarly with donor or recipient alone served as controls. Plates were incubated under standard conditions and examined periodically during a 7- to 9-day period for changes in morphology of the recipient strain. Modified recipient mycelium farthest from the donor strains was sampled, cultured under standard conditions, and compared with cultures of the donor and recipient strains. Samples of the modified recipient were paired with the recipient strain in its original condition. If the recipient rapidly acquired the cultural characteristics of the sample, the sample was considered to have acquired one or more transmissible cytoplasmic agents.

Attempts were made to approximately fulfill Koch's postulates for the agents in EP-60 by transmitting them separately and together in 10-step cycles (Fig. 1) from the nuclear genetic background of EP-60, represented by EP-523, a second-generation type C SCI, to the nuclear genetic background of EP-6, represented by EP-516, a first-generation methionine-requiring type C SCI, and back to the nuclear genetic background of EP-60. Following transmission from appropriate SCI by hyphal anastomosis in chestnut, the agents were increased by growing the "infected" recipient strain in culture, passed into two generations of SCI, increased again by culturing, transmitted by hyphal anastomosis in chestnut to the other nuclear genetic background, increased, and passed into two generations of SCI in this background. Only SCI which themselves had been subjected to single-conidial isolation were used as donors and recipients. This ensured that they were homokaryotic, not mixtures of strains, and that their infection states had been correctly identified. Colony morphology, methionine-requirement, and SCI segregation pattern were used together to determine if transmission had occurred.

Strains tested for dsRNA were grown for 7–9 days on PDAmb overlaid with cellophane, as described by Anagnostakis and Day (3). Eight-gram (fr. wt.) samples were extracted by the method of Morris and Dodds (34). Extracts equivalent to 4.0 g fr. wt. were electrophoresed on 5% polyacrylamide gels, 80 mm long, for 12 hr at 6 mA per gel (23). Gels were overloaded intentionally to permit minor dsRNA components to be detected. After electrophoresis, gels were stained with propidium iodide for 1 hr. Fluorescing dsRNA bands were photographed through a Wratten #15 filter while the gels were being exposed to ultraviolet light. Susceptibility of the stained material to digestion by ribonuclease in high- and low-salt solutions was determined by the method of Dodds (13).

A broad concept of virulence in *E. parasitica*, virulence (sensu lato), was used throughout this study and defined as a strain's combined pathogenicity and fruiting capacity in American chestnut (17,20). This concept resembles Nelson's (35) concept of parasitic or pathogenic fitness and is an extension of the more conventional concept, virulence (sensu stricto), based on pathogenicity alone. The pathogenicity component of virulence was measured as square centimeters of canker area, and the fruiting capacity (stromatal and perithecial) components as numbers of stromata and numbers of stromata containing perithecia, respectively. One square centimeter of canker area, 10 stromata, and 10 stromata containing perithecia were each assigned one unit of virulence. Strains were inoculated into smooth bark of 10- to 16-cm-diameter American chestnut trees in late spring. A 4 × 4 Latin square design was used. Canker area and number of stromata were determined 2 mo after inoculation, and canker area, numbers of stromata, and numbers of stromata containing perithecia 12 mo after inoculation. Three standard strains, EP-155, 408, and 421, were included in the experiment to provide a measure of normal virulence. Relative virulence, V_R, expressed as a percentage of normal virulence, was calculated by using the equation,

$$V_R = 100[(P_t + S_t + Pt_t)/(P_s + S_s + Pt_s)],$$

in which P_t, S_t, Pt_t are the mean pathogenicity, stromatal, and perithecial components of virulence for the test strain, and P_s, S_s, and Pt_s are corresponding means for the standard strains.

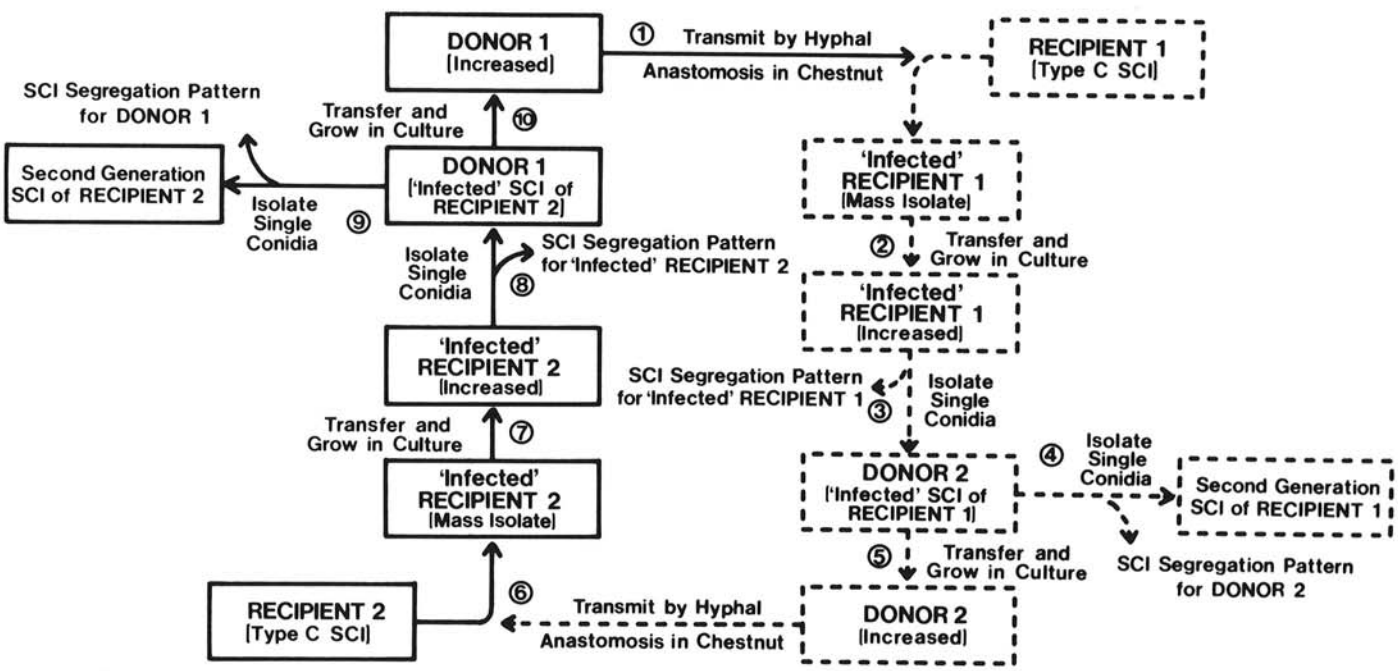

Fig. 1. Diagrammatic representation of the 10-step transmission cycles used to approximately fulfill Koch's postulates for the debilitating cytoplasmic agents from *Endothia parasitica* strain EP-60. The nuclear genetic background of EP-60 is represented by solid lines and includes Donor 1 and Recipient 2, and the methionine-requiring genetic background is represented by dashed lines and includes Recipient 1 and Donor 2. Donors and recipients were first- or second-generation single-conidial isolates (SCI) that had been subjected to single-conidial isolation to confirm their isolate types and to ensure they were not heterokaryons or mixtures of strains. Agents were transmitted between nuclear genetic backgrounds by hyphal anastomosis in bark of excised dormant American chestnut (steps 1 and 6). Within a background, they were transmitted through conidia (steps 3, 4, 8, and 9) for "purification," to determine SCI segregation patterns, and identify infection states. Remaining steps were for increase of fungus and agent(s) (steps 2, 5, 7, and 10).

To determine if the agents in EP-60 had curative effects, naturally occurring cankers on eight American chestnut trees in a wooded area were treated with a slurry (28) composed of mascerated cultures of strains containing both agents and representing 24 v-c groups. In June, 10-mm-diameter plugs of infected bark were removed with a cork borer at 1- to 2-cm intervals around the periphery of each canker. The holes were filled with the slurry and masking tape was applied. Untreated cankers on eight other American chestnut trees in the same area served as controls. Cankers were examined at irregular intervals for 2 yr after treatment.

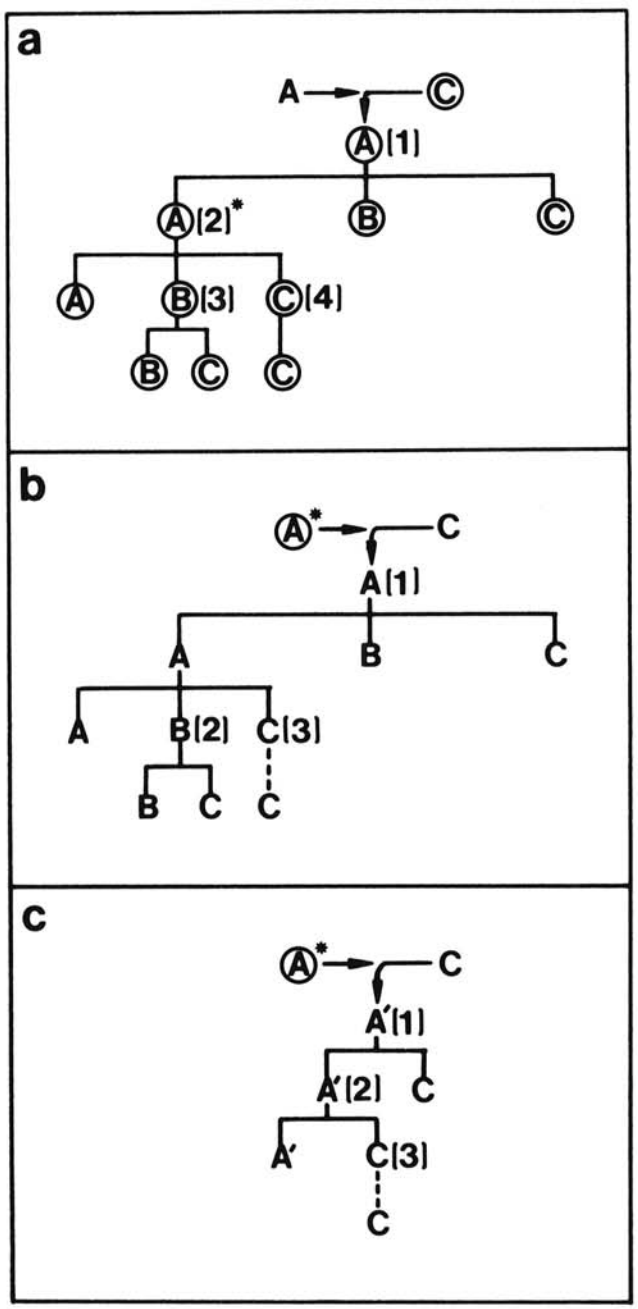

Fig. 2. Cyclic transmission of the agents for infection state A, **a,** from the nuclear genetic background of *Endothia parasitica* strain EP-60 (uncircled letters) to an unrelated methionine-requiring nuclear genetic background (circled letters) by hyphal anastomosis (arrows) and into two generations of single-conidial isolates (SCI), and **b,** from a first-generation methionine-requiring SCI (*) with infection state A back to the nuclear genetic background of EP-60 by hyphal anastomosis and into two generations of SCI. **c,** Repeating dichotomous SCI segregation pattern for a second mass isolate with type A cultural characteristics and infection state A′ (A′ [1]) from the transmission shown in **b**. Numbers in parentheses represent specific strains referred to in the text. Dashed lines between generations of type C isolates **(b and c)** represent tests made with spore masses.

RESULTS

Cyclic transmission of cytoplasmic agents. Fig. 2 summarizes the cyclic transmission of cytoplasmic agents in an SCI with cultural type A and infection state A. In the first half of the 10-step cycle, hyphal anastomosis between the donor and the methionine-requiring recipient yielded methionine-requiring mass isolates with type A cultural characteristics (Fig. 2a, [1]). The isolate tested yielded SCI with type A, B, and C cultural characteristics, i.e., a type A SCI segregation pattern. A first-generation, methionine-requiring SCI with type A cultural characteristics (Fig. 2a, [2]) also yielded a type A SCI segregation pattern. Second-generation type B and C SCI (Fig. 2a, [3] and [4], respectively) yielded SCI with type B and C and type C cultural characteristics, respectively, i.e., type B and type C SCI segregation patterns, respectively. In the second half of the 10-step cycle, pairing the first-generation, methionine-requiring SCI with type A cultural characteristics and infection state A (Fig. 2a, [2]) with EP-523 yielded prototrophic mass isolates with type A cultural characteristics. The first of these isolates tested (Fig. 2b, [1]) yielded type A SCI segregation patterns through two generations. A second-generation type B SCI (Fig. 2b, [2]) yielded a type B SCI segregation pattern, and the 30 spore masses from second-generation type C SCI (Fig. 2b, [3]) yielded only type C colonies. A second prototrophic isolate with type A cultural characteristics (Fig. 2c, [1]) produced only SCI with type A and C cultural characteristics, i.e., it had the predicted (21) fourth SCI segregation pattern and infection state. A. first-generation SCI with type A cultural characteristics (Fig. 2c, [2]) also produced this dichotomous pattern of SCI segregation. Thirty spore masses from second-generation type C SCI yielded only type C colonies. Isolates with type A cultural characteristics and the dichotomous pattern of SCI segregation were designated cultural type A, infection state A′.

Figs. 3 and 4 summarize the cyclic transmission of cytoplasmic agents in SCI with infection states A′ and B, respectively. In both nuclear genetic backgrounds, SCI segregation patterns for mass isolates with infection states A′ and B (Fig. 3a [1], 3b [1], 4a [1], and 4b [1], respectively) were dichotomous through two generations. In each case, spore masses from second-generation type C SCI (Figs. 3a [3], 3b [3], 4a [3], and 4b [3]) produced only type C colonies.

Pathogenicities were determined for the first-generation methionine-requiring SCI with infection state A (Fig. 2a, [2]), three each of its cultural type A, B, and C SCI, and EP-516. After 2 mo,

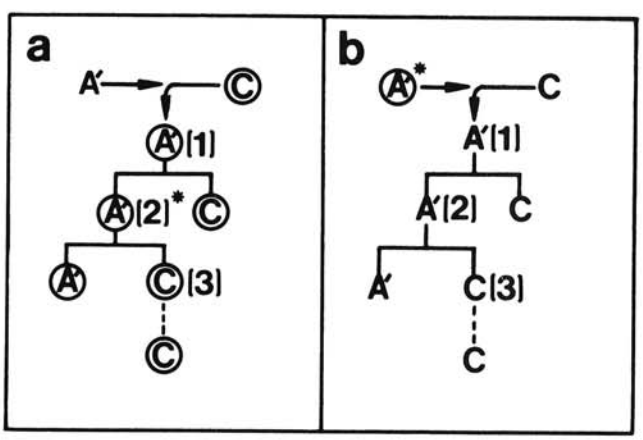

Fig. 3. Cyclic transmission of the cytoplasmic agent for infection state A′, **a,** from the genetic background of *Endothia parasitica* strain EP-60 (uncircled letters) to an unrelated methionine-requiring nuclear genetic background (circled letters) by hyphal anastomosis in chestnut (arrows) and into two generations of single-conidial isolates (SCI), and **b,** from a first-generation methionine-requiring SCI (*) with infection state A′ back to the nuclear genetic background of EP-60 by hyphal anastomosis and into two generations of SCI. Numbers in parentheses represent specific strains referred to in the text. Dashed lines between generations of type C isolates represent tests made with spore masses.

none of the cultural type A SCI had produced a canker, the type B SCI had produced small cankers (7.6 ± 0.5 cm^2), and the type C SCI and EP-516 had produced large cankers (29.5 ± 1.3 cm^2 and 30.1 ± 2.8 cm^2, respectively).

Pathogenicities also were determined for first-generation SCI with infection state A′ in the EP-60 and EP-6 nuclear genetic backgrounds (Fig. 3b, [2], and Fig. 3a, [2], respectively) and first-generation SCI with infection state B in these backgrounds (Fig. 4b, [2] and 4a, [2], respectively). Three months after inoculation, neither of the type A′ isolates had produced a canker, and the type B isolates had produced small cankers (6.4 ± 0.4 cm^2).

Transmission to diverse strains of *Endothia parasitica*. Experiments were made to determine if the abnormalities of the simplest segregating types, A′ and B, could be transmitted in culture to 20 diverse nuclear genetic backgrounds of *E. parasitica*, represented by the American strains, EP-42, 144, 155, 305, 366, 393, 502, 589, and 709, and Italian strains EP-46, 62, 65, 67, 408, 409, 421, 427, 432, 462, and 505. Type A′ and B forms of the EP-60 and EP-6 nuclear genetic backgrounds were used as initial donors. Transmission was easily recognized by a change of recipient mycelium to cultural type A or B, even with EP-393, the cream-colored mutant. Ease of transmission depended on the strains involved, as described by Anagnostakis and Day (3), Anagnostakis (2), and Kuhlman (31). Recipient strains not converted were paired again with the initial donors and with transmission products from the first experiment. This procedure was repeated until cultural type A and B forms of all 20 recipients were obtained.

In single-conidial isolation experiments, cultural type A and B forms of three American and four Italian recipient strains (EP-144, 155, and 589, and EP-408, 421, 432, and 505, respectively) produced cultural types A and C and B and C SCI, respectively. These seven pairs of infection state A′ and B forms, a set of infection state A′ and B forms from EP-60, and the eight corresponding type C forms were tested for pathogenicity. Two months after inoculation, none of the type A′ forms had produced a canker, the type B forms had produced small cankers (8.9 ± 0.9 cm^2), and the type C forms had produced large cankers (56.2 ± 3.8 cm^2).

Infection state A′ and B forms of the EP-6, 60, 155, 408, and 421 nuclear genetic backgrounds were paired in culture to determine if infection state A could be produced in each background. For the background of EP-60, the first-generation type A′ and B SCI shown in Figs. 3b [2] and 4b [2], respectively, were used. These two isolates represent this nuclear genetic background when it contains the more debilitating and the less debilitating agents, respectively, after they had separately traversed the 10-step transmission cycle. Over a 3- to 9-day period, the type B forms gradually acquired type A cultural characteristics. Mass isolates with type A characteristics, taken from the type B side of all five nuclear genetic backgrounds, yielded SCI with type A, B, and C cultural characteristics. First-generation SCI with type A cultural characteristics in the EP-6 and EP-60 backgrounds were also tested and yielded cultural type A, B, and C SCI.

All other nuclear genetic backgrounds of *E. parasitica* represented in Table 1 developed type A cultural characteristics when paired with appropriate infection type A forms in chestnut or in culture.

Proportions of cultural types produced in single-conidial isolation tests. Isolates with infection states A, A′, B, and C produced different SCI cultural types in different proportions (Table 2).

dsRNA. Strain EP-60 and infection states A, A′, B, and C of EP-155, 408, 421, and 523 (Fig. 5) were tested for dsRNA. The type

TABLE 2. Frequencies of cultural types among single-conidial isolates (SCIs) of strain EP-60 of *Endothia parasitica* and its derivatives[a]

Infection state	Total isolates tested and (SCI) (no.)	Frequencies of SCI cultural types (%)		
		A	B	C
A	14 (4,502)	11	77	12
A′	13 (3,999)	12	0	88
B	15 (3,766)	0	92	8
C	5 (1,288)	0	0	100

[a] Conidia from 2-wk-old colonies were suspended in sterile distilled water, dilutions (to yield 30-60 colonies per plate) were plated on complete medium (36), and these were incubated for 48 hr at 24–26 C. Germlings were transferred individually to PDAmb, grown 7–9 days at 20 C and with a 16-hr photoperiod, and scored for morphological type: A, like strain EP-60 with colonies slow-growing, asymmetrical, with little aerial mycelium; B, colonies symmetrical with growth rate and aerial mycelium between those of types A and C; C, colonies rapidly growing, symmetrical, with abundant aerial mycelium.

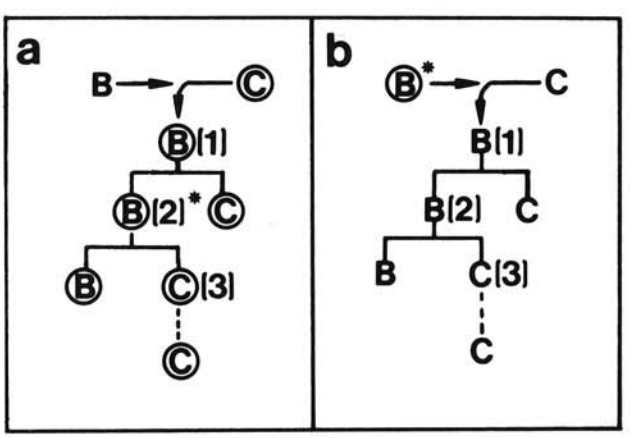

Fig. 4. Cyclic transmission of the cytoplasmic agent for infection state B, **a,** from the nuclear genetic background of *Endothia parasitica* strain EP-60 (uncircled letters) to an unrelated methionine-requiring nuclear genetic background (circled letters) by hyphal anastomosis in chestnut and into two generations of single-conidial isolates (SCI), and **b,** from a first-generation methionine-requiring SCI (*) with infection state B back to the background of EP-60 by hyphal anastomosis and into two generations of SCI. Numbers in parentheses represent specific strains referred to in the text. Dashed lines between generations of type C isolates represent tests made with spore masses.

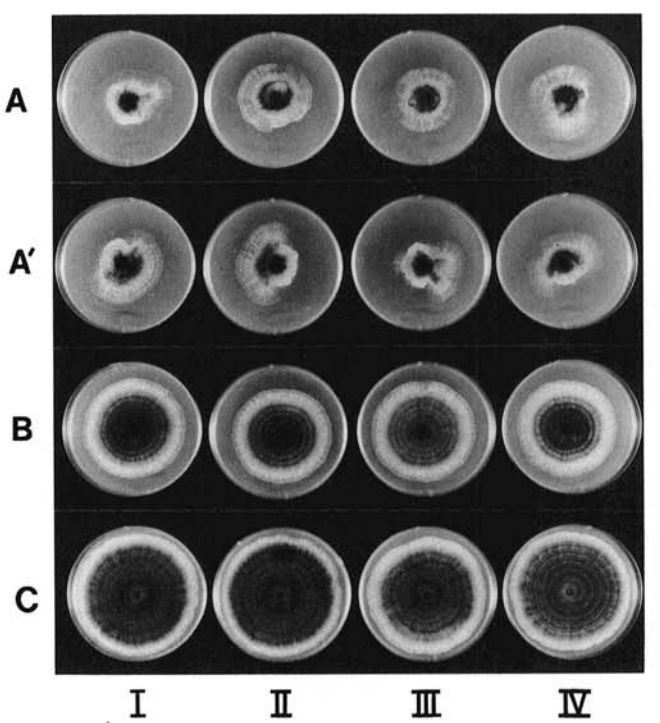

Fig. 5. Seven-day-old cultures of infection states A, A′, B, and C of three standard strains, EP-155 (I), 408 (II), 421 (III), and EP-523 (IV), a second-generation type C SCI of EP-60, grown on PDAmb at 20 ± 2 C with a 16-hr photoperiod.

A forms were those obtained by pairing the infection state A′ and B forms in culture. Infection states for all of the strains were confirmed by single-conidial isolation. Fig. 6 shows the patterns of dsRNA components found in extracts of the type A, A′, and B infection states of these strains and in a composite sample of each type. Composite samples were made by combining equal amounts of mycelium of the type A, A′, or B forms of the four nuclear genetic backgrounds before extraction. No dsRNA was detected in extracts from the type C forms (not shown). One major, three intermediate, and at least eight minor components were found in the type A′ isolates and the composite; one intermediate and at least eight minor components in type B isolates and the composite; and two partially resolved major bands, or a major and an intermediate band, three intermediate bands and ten minor bands in the type A forms, including EP-60 and the composite. (The minor bands in each set of gels were more easily discerned in the photographic negatives than in the prints.) Although the band intensities differed from strain to strain within an infection state, the patterns and relative band intensities appeared to be the same for each set of four strains and composite. The amount of dsRNA extracted from EP-60 and the other isolates with infection state A appeared to be higher than from isolates with either infection state A′ or B. Also, the pattern of bands for infection state A appeared to be a combination of the patterns for infection states A′ and B. The fluorescent material in the gels was not digested by ribonuclease in 0.3 M NaCl; in water, however, ribonuclease digested all of it.

TABLE 3. Relative virulences[a] of infection states A, A′, B, and C of the nuclear genetic background of EP-60 and three standard strains of *Endothia parasitica* 2 mo and 12 mo after inoculation into American chestnut trees

Nuclear genetic background	Infection state							
	A		A′		B		C	
	2 mo	12 mo	2 mo	12 mo	2 mo	12 mo	2 mo	12 mo
155	0	0	0	0	15	25[b]	89	81
408	0	0	0	0	11	5	118	124
421	0	0	0	0	13	8	95	95
523	0	0	0	0	9	3	89	107

[a] Relative virulence $V_R = 100\,[(P_t + S_t + Pt_t)/(P_s + S_s + Pt_s)]$ in which P_t, S_t, and Pt_t are the mean pathogenicity, stromatal, and perithecial components of virulence for a test strain; and P_s, S_s, and Pt_s are corresponding means for the standard strains (EP-155, 408, and 421) in their uninfected forms. One square centimeter of canker area, 10 stromata, and 10 stromata containing perithecia were each assigned one unit of virulence. Means for four inoculations per strain were used.

[b] This value is significantly greater than the others in this column ($P = 0.05$).

Virulence and relative virulence. The strains tested for dsRNA (Fig. 5) were tested for virulence (Fig. 7). The relative virulences of infection states A, A′, and C of the four nuclear genetic backgrounds, whether measured 2 or 12 mo after inoculation, did not differ significantly within a type ($P = 0.05$) (Table 3). However, by the criteria used, the infection state B form of EP-155 was significantly more virulent, when measured 12 mo after inoculation, than the other type B forms. These larger cankers were superficial, and only cultural type B forms were reisolated from their margins. All type A and A′ forms were without virulence, and type B forms were significantly less virulent than type C.

Treated cankers. Of eight cankers treated with the slurry containing the cytoplasmic agents from EP-60, seven ceased to enlarge within 1 mo after treatment. These cankers remained under control for the 22-mo duration of the experiment. Heavy callus developed at the canker margin, lifted the infected bark away from

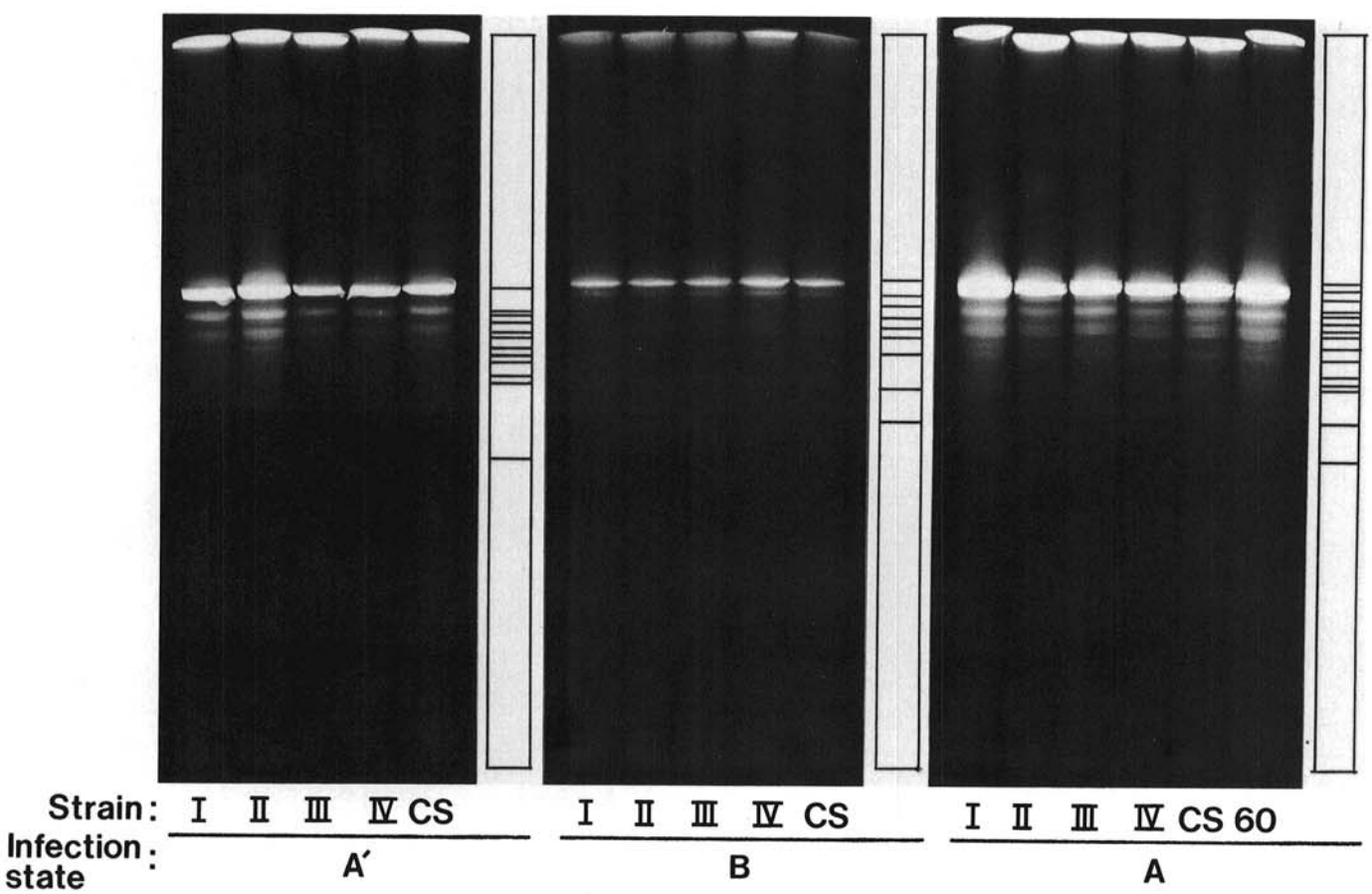

Fig. 6. PAGE gels showing patterns of dsRNA components extracted from infection states A′, B, and A of *Endothia parasitica* strains EP-155 (I), 408 (II), 421 (III), 523 (IV), a composite sample of I through IV (CS), and from EP-60 (V). Infection states C (not shown) yielded no detectable dsRNA. Extracts equivalent to 4.0 g fr. wt. were electrophoresed on 5% gels for 12 hr at 6 mA per gel. Gels were stained with propidium iodide and photographed while being exposed to ultraviolet light. The diagram to the right of each photograph depicts the pattern of fluorescing bands detectable in the negative.

the sapwood, then the bark gradually split and fell away (Fig. 8). All of the untreated control trees died within the 2-yr period of the experiment.

DISCUSSION

Together, the findings of this and the earlier study (21) support eight conclusions about strain EP-60: (i) its nuclear genetic background is typical of *E. parasitica*, conferring normal cultural characteristics and a normal level of virulence in American chestnut; (ii) two independent, debilitating cytoplasmic agents confer the abnormalities of EP-60; (iii) when present alone, each agent confers different cultural abnormalities and a different level of hypovirulence; (iv) when present together, the more debilitating agent determines cultural characteristics and virulence; (v) in culture, the agents are transmitted into conidia with different frequencies; (vi) with a minor exception, each agent confers its associated abnormalities in many other nuclear genetic backgrounds of *E. parasitica*, whether from North America or Italy, whether or not they had been infected previously then freed of other debilitating cytoplasmic agents, and regardless of v-c group; (vii) a different pattern of dsRNA components is associated with each agent; and (viii) together the agents have marked curative effects when introduced into naturally occurring blight cankers on American chestnut trees.

The properties of EP-523, a second-generation type C SCI of EP-60 (21), indicate that EP-60 is a strain of *E. parasitica* with a normal nuclear genetic background. EP-523 lacked detectable dsRNA, had cultural characteristics like those of the standard strains (Fig. 5), produced perithecia and ascospores typical of *E. parasitica* both in the field and when crossed with mating type testers in the laboratory (21), and it was as virulent in American chestnut over a 12-mo period as the three standard strains (Fig. 7, Table 3). Also, the agents from EP-60 produced similar changes in the cultural characteristics and virulence of EP-523 and the three standards (Figs. 5 and 7).

The evidence that two independent, debilitating cytoplasmic agents confer the abnormalities of EP-60 is entirely correlative, but it is extensive, consistent, and has no other obvious interpretation. The results of the 10-step cyclic transmission experiments provide the strongest evidence, because they approximately fulfill Koch's postulates (as they apply to obligate parasites) for the agents separately and together. The consistent effect each agent had on the cultural characteristics and pathogenicity of both nuclear genetic backgrounds used in the cycle approximately fulfills the first three of Koch's postulates. The fourth postulate was approximately fulfilled when the agents were transmitted back to the original nuclear genetic background, typical disease symptoms developed, and the products of transmission yielded typical patterns of SCI segregation. The main obstacle to fully satisfying Koch's postulates is our inability to observe and manipulate the agents directly. Dodds (11) examined EP-60 for viruslike particles, using the procedure that yielded membrane-bound "particles" from European strains (12), but he found none. Thus, with current knowledge, presence of the agents from EP-60 can only be inferred from the symptoms they produce in their host and the patterns of dsRNA components that can be extracted from the host.

Other evidence also indicates that two independent cytoplasmic agents are involved. Four infection states designated A, A′, B, and C (Fig. 5) were derived from strain EP-60 (infection state A) and established in each of two other North American and two Italian nuclear genetic backgrounds of *E. parasitica*. Four infection states (N) are expected when two independent cytoplasmic agents (n) are present ($N = 2^n = 2^2 = 4$). Dominance of the more debilitating agent when both agents are together accounts for the discrepancy

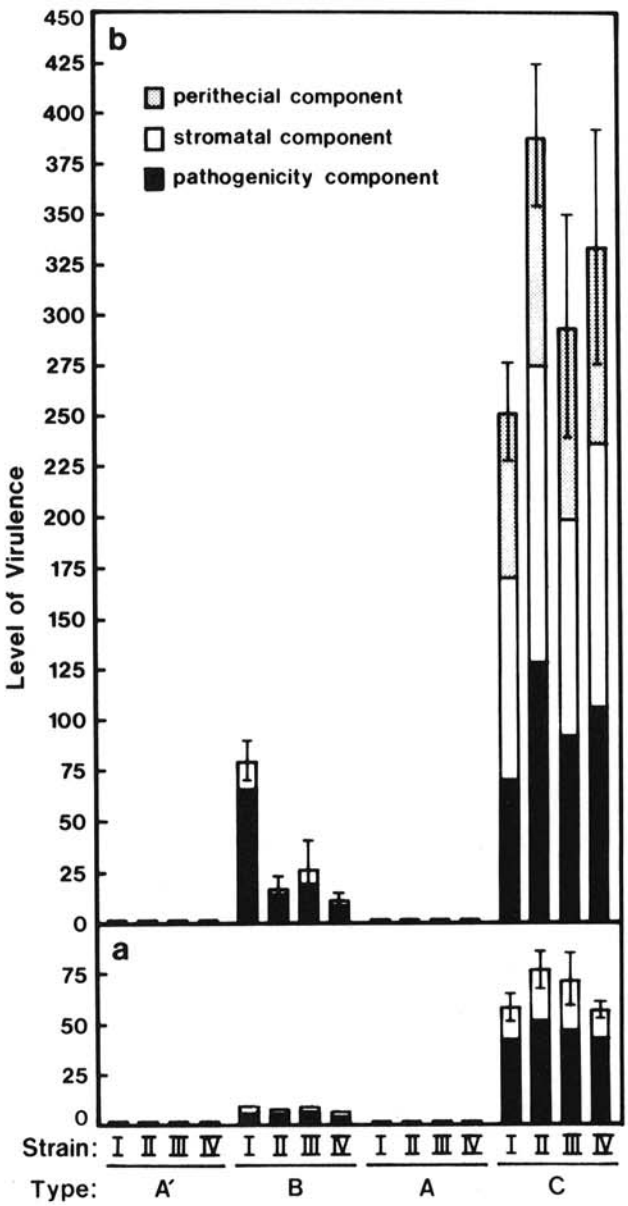

Fig. 7. Estimated virulences of infection states A′, B, A, and C of EP-155 (I), 408 (II), 421 (III), and 523 (IV) **a,** 2 mo and **b,** 12 mo after inoculation into each of four American chestnut trees. Virulence was calculated as the sum of the mean canker area in square centimeters, the mean number of stromata divided by 10, and the mean number of stromata containing perithecial beaks divided by 10. Bars represent standard errors.

Fig. 8. Chestnut blight cankers 22 mo after treatment with a slurry of strains containing the cytoplasmic agents from *Endothia parasitica* strain EP-60. The slurry was applied in June in holes made with a cork borer at 1- to 2-cm intervals about the margin of each canker.

between the number of cultural types (three) and the number of infection states (four). The dichotomous SCI segregation patterns found for infection states A′ and B indicated that each contained one agent ($N = 2^1 = 2$). The lack of segregation of SCI from type C agrees with the prediction for $n = 0$ ($N = 2^0 = 1$). Restoration of the type A infection state in five nuclear genetic backgrounds, following hyphal anastomosis between the type A′ and B infection states, provides further evidence that strain EP-60 contains two independent agents. The evidence obtained with the nuclear genetic background of EP-60 is especially compelling because, in this background, each agent had independently traversed the 10-step cycle before infection state A was restored. The segregation of SCI from this type A isolate into the three cultural types through two generations can only be explained by the presence of two cytoplasmic agents.

The consistent, but distinct, patterns of dsRNA components obtained from the type A′ and B infection states of the nuclear genetic backgrounds of EP-60, the three standards, and the composite samples, and the apparent combination of the amounts and patterns of dsRNA components in the corresponding type A infection states add substantially to the evidence for two agents. Studies of RNA hybridization (33) would be required to determine the degree of relatedness, if any, of the two agents.

Finally, the distinct transmissibilities of the two agents into conidia, whether or not the other agent was present (assuming that most cultural type A SCI from a strain with infection state A contain both agents) (Table 2), supports the conclusion that two independent agents are present.

The low transmissibility of the more debilitating agent into conidia, the high transmissibility of the less debilitating agent, and the indistinguishable cultural characteristics and pathogenicities of types A′ and A, account for the difficulty of obtaining and recognizing type A′ SCI. The type A′ and A infection states were distinguishable only by their patterns of SCI segregation and dsRNA components.

The consistent effects the agents from EP-60 had on the cultural characteristics of a wide variety of North American and Italian nuclear genetic backgrounds of *E. parasitica* supports the suggestions (20) that the populations of the fungus in North America and Italy are similar and relatively uniform, except perhaps for diversity of v-c groups (4), and that the diverse characteristics of the dsRNA-containing strains may be due more to diversity among dsRNA-associated agents than among nuclear genetic backgrounds of the fungus. Likewise, the typical effects the agents had in the seven type C SCI of North American and Italian dsRNA-containing strains suggests, as have other studies (8,10,26), that such isolates are normal. This is also supported by results of an experiment in which seven such isolates of diverse origin and a dsRNA-free mass isolate were inoculated into American chestnut trees in the field, where they were exposed for 16 mo to the stresses imposed by interactions with host tissue and changing weather conditions. During this period, all of the strains girdled the trees and produced abundant stromata and perithecia, and only normal-appearing forms were reisolated (*unpublished*).

The consistent changes in cultural characteristics produced by the agents from EP-60 contrasts with results reported for cytoplasmic determinants from some European strains (1,3) but not others (19). Mixtures of cytoplasmic agents or fundamental differences in the biology of the agents may account for these differences. The significance of the atypical effect the less debilitating agent from EP-60 had on the virulence of strain EP-155 is unclear.

The slurry of strains containing both CH agents from EP-60 effectively controlled naturally occurring chestnut blight cankers on American chestnut trees. The relative effectiveness of the two agents in controlling cankers remains to be determined.

The strategy used to investigate the causes of hypovirulence in EP-60 extends the approaches that Grente and Sauret (26) and Bonifacio and Turchetti (8) used to study abnormal isolates from recovering European chestnut trees. In all three approaches, single-conidial isolation was used effectively to free a strain's nuclear genetic background of cytoplasmic agents and to determine patterns of SCI segregation, operations made possible by the minute size and uninucleate condition of *E. parasitica* conidia (5,8). In this study, comparison of the pathogenicities of the various categories of SCI and further single-conidial isolation experiments permitted most of the basic isolate types to be obtained and identified. However, the elusive fourth infection state, A′, was obtained unexpectedly as a product of transmission by hyphal anastomosis. Contributions made to the virulence of the test strain by its nuclear genetic background and cytoplasmic agents were assessed by comparing the virulence of the test strain, with and without the agents, with the virulences of the three standard strains, with and without the agents, in the same trees. The standard strains, when free of the agents, provided a measure of normal virulence, which was used to determine both the relative level of virulence conferred by the test strain's nuclear genetic background and the extent to which the cytoplasmic agents changed the levels of virulence conferred by the nuclear genetic backgrounds of the test strains and the standard strains. The rationale for using the broad concept of virulence with *E. parasitica* has been discussed (18,20).

A convenient system is needed for designating nuclear genetic backgrounds and CH agents. A modification of the system used by Anagnostakis (1) has been proposed (18) in which the nuclear genetic background of a strain is designated by the culture collection number of its dsRNA-free state, the infection state of its cytoplasm is indicated in brackets following this number, and CH agents are represented by symbols of the form H_{Xn}, in which X = geographic origin and n = agent number. The equivalencies for infection states of EP-60 are as follows: infection state A = 523 [$H_{M1} + H_{M2}$]; infection state A′ = 523 [H_{M1}]; infection state B = 523 [H_{M2}]; and infection state C = 523 [ ].

The availability of defined systems such as derived from EP-60, especially sets of standard strains containing individual CH agents, makes possible more meaningful studies of the identity, relatedness, molecular biology, and, perhaps, the sources of the CH agents that now occur in *E. parasitica*. This knowledge should lead to a better understanding of the natural recovery and CH phenomena and, perhaps, to their more effective application in the control of chestnut blight.

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