Preliminary Evidence for Two Debilitating Cytoplasmic Agents in a Strain of *Endothia parasitica* from Western Michigan

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ABSTRACT

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Single-conidial isolates (SCI) of EP-60, a highly debilitated, dsRNA-containing strain of *Endothia parasitica* from western Michigan, segregated into three types. Type A was indistinguishable from EP-60 in cultural characteristics, pathogenicity, and fruiting capacity in American chestnut, and in its pattern of SCI segregation. Type B had cultural characteristics unlike type A or typical *E. parasitica*, was weakly pathogenic, and produced a few stromata, perithecia, and ascospores typical of *E. parasitica* in American chestnut. It yielded only type B and C SCI. Type C was indistinguishable from typical *E. parasitica*. It produced typical perithecia and ascospores in the field and in the laboratory, when mated with a type *a* mating type tester, and it yielded only type C SCI. When type A, B, and C isolates were paired on agar, type A and B characteristics were rapidly transferred to type C, and type A characteristics were slowly transmitted to type B. These results indicate that EP-60 has a genetic background typical of *E. parasitica*, with mating type *A*, and suggest that it contains two debilitating cytoplasmic agents. Type B isolates appear to represent the genetic background of EP-60 when it contains one of the agents and type A this background when it contains both agents. A fourth isolate type, with only the second agent, was not found.

Additional key words: *Castanea dentata*, hypovirulence.

Development of swollen, often superficial, cankers is associated with natural recovery of chestnut (*Castanea* spp.) from chestnut blight, which is caused by *Endothia parasitica* (Murr.) P. and H. And. (6,8,15,17,20). These cankers usually contain normal and abnormal forms of the fungus (5,7,15). The abnormal forms typically contain dsRNA (9), which is the genetic material of many fungal viruses (18). Thus, natural recovery may be affected by pathogenic, viruslike agents that weaken the fungus. Little is known about the nature, diversity, biology, molecular biology, or origin of these agents (11,12).

In a previous study, characteristics of 32 European and North American dsRNA-free and dsRNA-containing strains were compared (13). The dsRNA-free strains, including single-conidial isolates (SCI) from three dsRNA-containing strains, had similar characteristics. In contrast, the dsRNA-containing strains differed from one another and from the dsRNA-free strains. Differences among dsRNA-associated agents may account for much of this diversity. Also, some strains may contain more than one such agent. The diverse characteristics of dsRNA-containing SCI from two Italian strains (13), among derivatives of French strains (16), and of a French and an Italian dsRNA-containing strain (3) support these possibilities. Based on differences in patterns and amounts of dsRNA, Dodds (10) concluded that the agents in European and American strains differ. Differences in cultural characteristics also support this conclusion (13). Together these observations suggest that detailed studies of individual strains will be needed for an understanding of the bases of this diversity.

The objective of this study was to determine the factors that contribute to the debilitation of EP-60, a nonpathogenic, dsRNA-containing strain isolated from recovering American chestnut in western Michigan. Results of SCI segregation, pathogenicity, and within-strain transmission tests are reported in this paper. Transmission of agents to and from unrelated genetic backgrounds, patterns of SCI segregation in these backgrounds, patterns of dsRNA components, and effects of the agents on virulence (sensu lato) (13) will be reported separately. A preliminary report has been given (14).

MATERIALS AND METHODS

Strain EP-60 (ATCC 38765); single-conidial isolates (SCI) of EP-60; EP-29, a dsRNA-free American mass-ascospore isolate (ATCC 38754); and mating type testers EP-42 (type *A*) (ATCC 38751) and EP-67 (type *a*) (ATCC 38753) were maintained and grown for inoculum and determination of cultural characteristics as described previously (13). Single-conidial isolation experiments (17) and determinations of pathogenicity (defined here as level of ability to colonize and kill host tissue) and fruiting capacity in excised dormant and intact American chestnut (13) also were made as described. Two hundred to 600 SCI per strain were prepared and evaluated for morphological type.

Single-ascospore isolates were made as follows: bark bearing stromata and perithecia were moistened with water, perithecia were teased from stromata with a fine needle, rolled over the surface of water agar to remove surface debris, and hydrated for about 10 min in a drop of sterile water on a flamed glass slide. Each intact perithecium was transferred to the inside wall of a test tube containing 10 ml of sterile water and gently crushed with a flamed glass rod. Perithecial contents with mature asci and ascospores were suspended by agitating the tubes with a Vortex mixer three times at approximately 5-min intervals. Each suspension was diluted serially from 1:10 to 1:1,000 with sterile water, and 1-ml aliquots of each dilution were spread on each of three plates of complete medium (CM) (19). Plates were incubated for 20–24 hr at 24–26 C, then approximately 50 germlings per perithecium were isolated, cultured, and evaluated for morphological types for SCI.

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Mating type was determined by the method of Anagnostakis (2), except American chestnut stem pieces were split into quarters and two quarters were spaced about 1 cm apart in each dish. This change confined each test strain to a separate stem piece during the colonization phase of the experiment.

RESULTS

Strain EP-60 consistently yielded what appeared to be three distinct types of first-generation SCI (Fig. 1, step 1). These types, designated A, B, and C, differed in cultural characteristics, pathogenicity, and types of SCI that each type produced in further SCI tests.

In culture, type A SCI (Fig. 2), like EP-60, were easily distinguished from the other types, but no two colonies were alike. They grew slowly (Fig. 3a) and asymmetrically, with most of the mycelium either closely appressed to the agar surface or embedded in it. The mycelium, which ranged from dark brown, through tan and yellow, to white, differed markedly in thickness and texture from one region to another. An irregularly shaped, dark brown, thickened area usually developed in the center. Leading mycelium in some regions was sparse, with individual hyphae growing in different directions; in others, it was more dense and grew in fanlike arrangements or as more parallel aggregates of hyphae. Pycnidia were minute, dark brown, and arranged in tightly packed masses on the thickest mycelium and in chainlike or feathery arrangements on moderately thick mycelium.

Type B isolates (Fig. 2) grew more rapidly (Fig. 3a) and produced radially symmetric, orange-brown colonies that had more aerial mycelium than type A. Leading mycelium was organized into mounds of densely packed, more or less parallel hyphae, alternating with less-dense mycelium, giving the margins a radially striated appearance. Pycnidia produced by type B were larger than pycnidia produced by type A isolates and were arranged in a nearly continuous convoluted mass near the colony center and in indistinct concentric rings nearer the margin.

Type C isolates (Fig. 2) were indistinguishable from typical wild-type isolates of *E. parasitica* (13). They grew most rapidly (Fig. 3a) and produced radially symmetric, orange colonies with abundant aerial mycelium, pronounced radial striations in the leading mycelium, and large, orange pycnidia arranged in concentric rings.

The differential pathogenicity of first-generation SCI was evident in short-term laboratory tests in which chestnut stem pieces harvested in March were used (Fig. 3b). Reisolation from cankers produced by type B and C SCI (eight sites per canker) yielded only type B and C isolates, respectively.

Second-generation SCI (ie, SCI from first-generation SCI) segregated as follows: type A (Fig. 1 [isolate 1]) yielded types A, B, and C (Fig. 1, step 2a); type B (Fig. 1, [isolate 2]) yielded types B and C (Fig. 1, step 2b); and type C (Fig. 1, [isolate 3]) yielded only type C (Fig. 1, step 2c). Growth in culture and short-term pathogenicities of second-generation SCI (Fig. 3c and d, respectively) resembled those of first-generation SCI. Use of stem pieces harvested in January probably accounts for the less extensive growth of second-generation type B and C SCI in chestnut.

Second-generation type B (Fig. 1, [isolate 4] and [isolate 6]) and type C SCI (Fig. 1, [isolate 5] and [isolate 7]) yielded third-generation type B and C SCI and type C SCI, respectively (Fig. 1, steps 3a, b, c, and d).

A long-term field test of pathogenicity and fruiting capacity was made with EP-60, first generation type A, B, and C SCI (Fig. 1, [isolate 1], [isolate 2], and [isolate 3]), and EP-29 (five replicate inoculations per isolate). Typical results 22 mo after inoculation are shown in Fig. 4. Inoculation with strain EP-60 and the type A SCI (Fig. 4b and c, respectively) produced the same injury as treatment with agar alone (Fig. 4a). The small cankers produced by the type B isolate ceased enlarging within 2 mo after inoculation (Fig. 4d). A few scattered stromata and a few perithecia developed in the colonized bark during the first growing season. The type C isolate and EP-29 sustained pathogenesis during both growing seasons, produced abundant stromata and perithecia both seasons, and girdled the trees during the second season (Fig. 4e and f, respectively). Perithecia produced by type B and C SCI, like those of EP-29, had the black necks and white bodies that are typical of *E. parasitica* (4). The ascospores produced were pigmented, two-celled, and constricted at the septum. Mean dimensions for 25 ascospores were 9.1×4.4, 8.8×4.2, and 8.3×3.8 μm for the type B and C SCI and EP-29, respectively. Of 425 single-ascospore isolates from nine perithecia on three cankers produced by the type B SCI, 417 had typical type C cultural characteristics. None of the remaining isolates resembled type A, B, or C.

First-generation type A, B, and C SCI were paired on agar to determine if type A and B characteristics were transmissible to type C and if type A characteristics were transmissible to type B. Plugs of inoculum were placed about 2 mm apart in the center of each plate of PDAmb and incubated under standard conditions. In pairings of type A or B with type C, the type C half of the colony acquired type A or B characteristics, respectively, within 2 or 3 days after inoculation. In pairings of type A with type B, the type B half of the colony gradually acquired type A characteristics over a period of 7 or more days.

In a test of mating type, a type C SCI (Fig. 1, [isolate 5]) produced perithecia and ascospores typical of *E. parasitica* when mated with the type *a* tester but not with the type *A* tester. Anagnostakis (1) assigned this type C isolate to vegetative compatibility (vc) group 9.

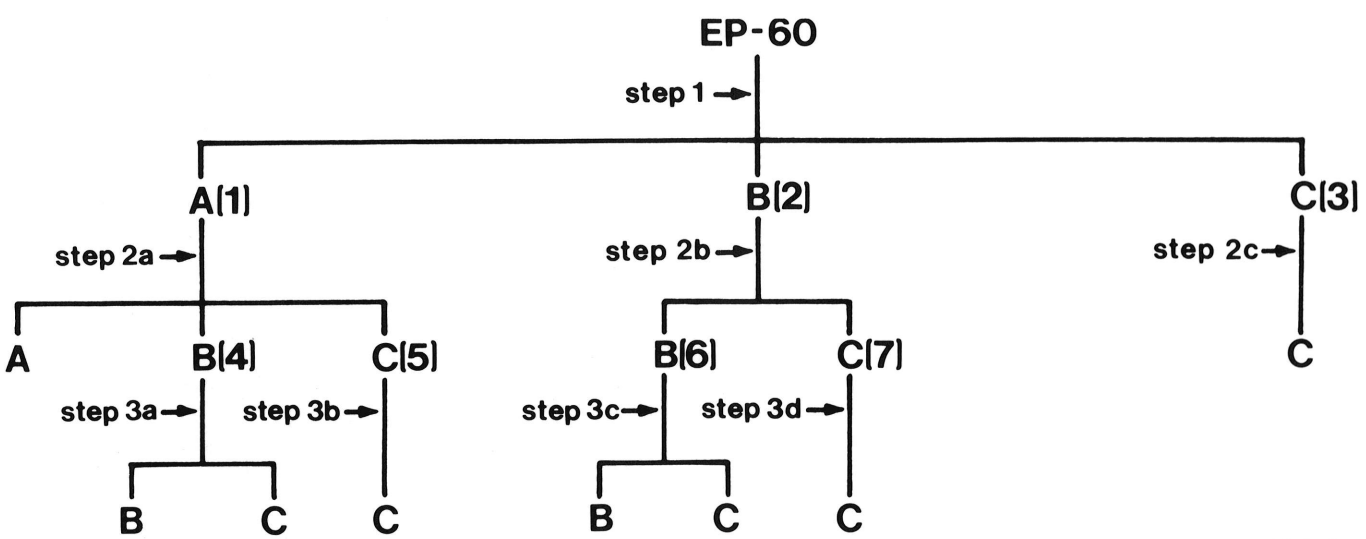

Fig. 1. Patterns of segregation of single-conidial isolates (SCI) from EP-60 and its type A, B, and C SCI. Numbers in parentheses represent specific isolates referred to in the text.

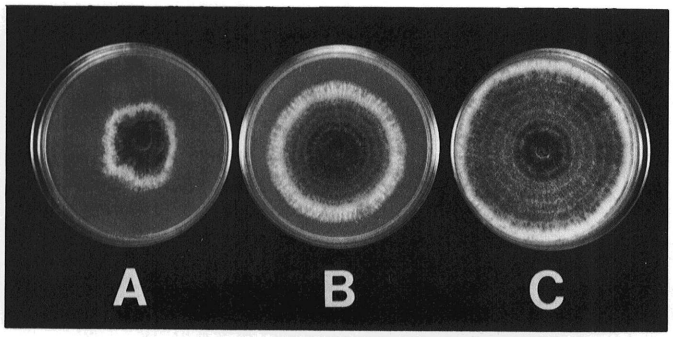

Fig. 2. Seven-day-old colonies of type A, B, and C single-conidial isolates of EP-60 grown on PDAmb at 20 ± 2 C with a 16-hr photoperiod.

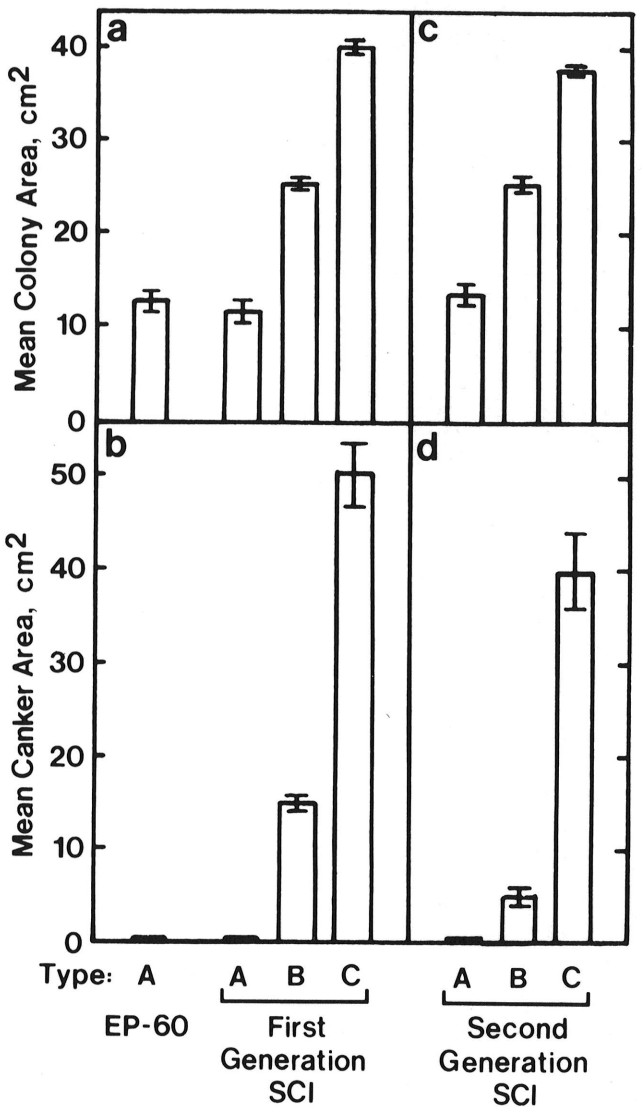

Fig. 3. Growth of EP-60 and representative first- and second-generation type A, B, and C single-conidial isolates (SCI) in culture and in excised dormant American chestnut. **a,** Mean colony areas ± standard error for EP-60 (three replicate colonies) and three each of its first-generation type A, B, and C SCI (three replicate colonies per isolate) after 7 days on PDAmb at 20 ± 2 C and a 16-hr photoperiod; **b,** mean canker areas ± standard error for EP-60 (four replicate inoculations) and three each of its first-generation type A, B, and C SCI (four replicate inoculations per isolate) 4 wk after inoculation; **c,** mean colony areas ± standard error for representative second-generation type A, B, and C SCI (three isolates per type and three colonies per isolate) under conditions given above; **d,** mean canker areas ± standard error for representative second-generation type A, B, and C SCI (three isolates per type and four inoculations per isolate) under conditions given above. First-generation SCI and EP-60 were tested in one experiment and second-generation SCI in another.

DISCUSSION

The results demonstrate that EP-60 has a typical genetic background of *E. parasitica*, with mating type *A*, vc group 9, and transmissible cytoplasmic agents that change its cultural characteristics and reduce its pathogenicity and fruiting capacity in chestnut. Because conidia of *E. parasitica* are uninucleate, the segregation of three types of SCI through two successive generations rules out the possibility that heterokaryosis (7) is responsible for the abnormalities. However, this pattern of segregation does suggest that two cytoplasmic agents are present.

The number of infection states, or isolate types, expected with n independent cytoplasmic agents is 2^n. With zero, one, and two

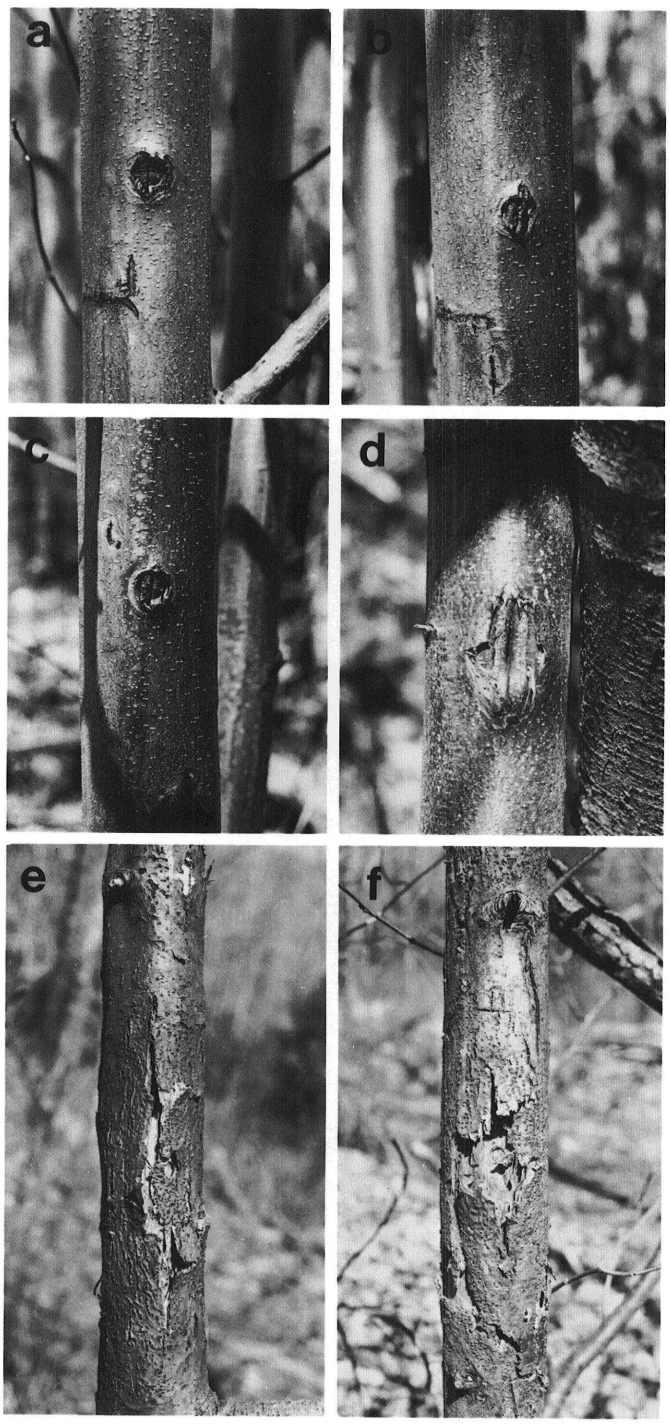

Fig. 4. Cankers and wound responses in American chestnut 22 mo after treatment with **a,** PDA; **b,** EP-60; **c,** first-generation type A single-conidial isolate (SCI) of EP-60; **d,** first-generation type B SCI; **e,** first-generation type C SCI; and **f,** EP-29.

agents, one, two, and four isolate types, respectively, are expected. The three types of SCI from EP-60, a number that fits none of these predictions, can be interpreted as follows: type C represents the genetic background of EP-60 when free of deleterious cytoplasmic agents; type B represents this genetic background when it contains one transmissible cytoplasmic agent, and type A, including EP-60, represents this genetic background when it contains the agent in B plus another. The fourth type predicted, which would contain only the second agent, was not detected.

The evidence for this interpretation is as follows: Type C had cultural characteristics, pathogenicity, fruiting capacity, perithecia, and ascospores typical of dsRNA-free *E. parasitica*. It was the only SCI type common to all SCI segregations and the only type whose SCI did not segregate. This fits the prediction for $n = 0$ agents. Also, when paired with type A or B SCI on agar, type C was readily converted to type A or B, respectively. Type B, with cultural characteristics, pathogenicity, and fruiting capacity between those of EP-60 and type C, and perithecia and ascospores typical of *E. parasitica*, segregated only type B and C SCI through two generations. A repeating dichotomous segregation such as this is expected when one cytoplasmic agent is present (2^1 = two types). The interpretation of EP-60 and type A SCI is based on the consistent segregation of their SCI into three types: A, B, and C. This could occur only if two agents were present. The absence of the fourth type predicted for two agents may be an illusion caused by the dominant effects of the more debilitating agent when the agents are together. That is, two infection types may have the cultural characteristics and pathogenicity of type A. One infection type may contain the agents for types A and B, and the other may contain only the agent for type A. These infection types should have different SCI segregation patterns. Two observations support the hypothesis that the effects of the agent for type A are dominant when both agents are present: the first is that EP-60 and the few type A SCI that were tested consistently produced type B SCI, and the second is that the type B SCI gradually changed to type A when paired in culture with type A. The fourth type, should have a dichotomous SCI segregation pattern like type B.

The agent for type B does not appear to enter ascospores; no type B single-ascospore isolates were found among the 425 isolates made from type B perithecia. This is consistent with earlier observations (9) and suggests that ascospore formation may be a mechanism by which *E. parasitica* can become free of debilitating cytoplasmic agents.

Additional evidence that EP-60 contains two independent, debilitating, dsRNA-associated, cytoplasmic agents will be presented separately.

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