NATURAL DISSEMINATION OF ARTIFICIALLY INOCULATED HYPOVIRULENT STRAINS OF *ENDOTHIA PARASITICA*

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ABSTRACT.--This study was designed to determine if hypovirulent (H) isolates of Endothia parasitica could be recovered from cankers that developed naturally on American chestnuts Castanea dentata previously inoculated with H strains. Isolates from 19 percent (89) of 475 cankers that developed on 220 trees over a 21/2-year period displayed morphological characteristics that were different from wild-type virulent (V) strains. These atypical isolates were separated into six morphological groups. A representative isolate from each group was able to alter the morphology of several V strains when grown together in vitro. Double-stranded RNA was detected in the representative isolates from four of the six groups. The results of these tests and their morphological similarity to known H strains indicate that isolates in at least four of the six groups are H. When cankers that yielded H isolates were extensively sampled, several different H types and V isolates were sometimes recovered from a single infection.

Introduction

Hypovirulent (H) strains of the chestnut blight pathogen, *Endothia parasitica*, are believed to be responsible for the natural control of chestnut blight that has occurred on European chestnuts, *Castanea sativa*, throughout Italy (Grente and Berthelay-Sauret 1978; Turchetti 1978) and on American chestnuts, *Castanea dentata*, in several areas of Michigan (Weidlich this proceedings). They have been used to biologically control cankers incited by wild-type virulent (V) strains (Van Alfen et al. 1975; Grente and Berthelay-Sauret 1978). Establishment of a self-perpetuating H based control process within the native range of the American chestnut would require that H strains disseminate throughout the existing V population of *E. parasitica* (Day 1978). Large abnormal cankers, found in areas where remission of chestnut blight has been observed, could be important to dissemination by providing reservoirs of persistent H inoculum (Elliston 1982). This paper reports on attempts to artificially establish large H cankers on American chestnuts and determine if H strains were present in naturally occurring cankers that developed on the same trees.

Materials and Methods

In May, 1979, two 0.1 hectare study plots with abundant American chestnut regeneration were established in 10- to 15-year-old cut-over areas near

Parsons and Bartow, West Virginia. Chestnut stems ranged in size from 3 to 11 cm, 1.3 m above the ground. The plots were cleared of competing vegetation and trees with visible *E. parasitica* cankers removed leaving uninfected individuals for the study.

Inoculum preparation and inoculation procedure. Eleven hypovirulent (H) strains of *E. parasitica* representing collections from France, Italy and the United States were used as inoculum (Table 1). Each strain was grown at 25 C in two 1000 ml Erlenmeyer flasks that contained 300 ml semi-solid glucose yeast extract (GYE) agar medium. The GYE medium contained 10.0 g glucose, 2.0 g Difco Bacto yeast extract, 1.0 g of KH2P04, 0.5 g MgS04•7H20, 0.2 mg of Zn++, 0.1 mg of Mn^{+}+, 0.1 mg of thiamine hydrochloride, 0.01 mg of biotin, 4.0 g of Difco Bacto agar, and 1000 ml of distilled H20 (Lilly and Barnett 1951). After 10 days growth, the contents of both flasks were combined and mixed in a waring blender to make an agar slurry.

Table 1. Hypovirulent strains of *Endothia parasitica* inoculated into wounded American chestnuts

Strain origin	Strain designation
France	JR 4[a/], 43[b/], A-1[b/]
Italy	27-10[a/], 54-2[a/], 50[a/], Euro 11[c/], Euro 7[c/]
United States	60[a/], 209[a/], 206[a/]

a/ Obtained from the Connecticut Agricultural Experiment Station, New Haven, CT 06504

b/ Isolated from virulent cankers on American chestnuts inoculated with hypovirulent strains; *in vitro* characteristics resembling French hypovirulent strains (Grente and Berthelay-Sauret, 1978)

c/ Isolated from abnormal cankers on European chestnuts in Italy

Twenty-five cm wounds encircling half the circumference of the study trees were made by "scratching" the bark with metal wood screws mounted in a wooden board. Wounds were made at ground level, 1.5 m and 3.0 m above the ground on each tree. The same individual hypovirulent strain was introduced into all three wounded areas on a tree by spreading the agar slurry over the wounds with a 6 to 8 cm wide brush. The wounds were covered with clear plastic (0.5 mil) and sealed with masking tape for approximately 30 days. All strains were inoculated into 10 replicate trees in each study plot so that a total of 220 trees were used.

Isolations. When isolations of mycelium were made from cankers, 0.5 cm diameter bark plugs were removed with a leather punch. Plugs were surface sterilized in a 0.5 percent sodium hypochlorite solution for 10 minutes,

washed in distilled H20 and placed in petri plates containing GYE medium amended with 2 percent agar, streptomycin (6 mg/1) and chlorotetracycline (50 mg/1). Mycelial transfers were made from the advancing edge of cultures from each plug and placed in petri plates containing Difco potato dextrose agar medium (PDA) amended with biotin (5 pg/1) and methionine (100 mg/1). Two transfers were placed at maximum spacing on each plate, incubated at 25 C in a 16-hr photoperiod (white fluorescent light) for 7 to 10 days and then morphologically evaluated.

Canker survey and isolate designations. Trees in both study plots were surveyed every 60 days between April and December for the presence of *E. parasitica* cankers in nonwounded areas. When cankers were initially observed, isolates of *E. parasitica* were obtained from five different points and classified as morphologically resembling wild-type V strains (Elliston 1978) or showing atypical characteristics. Atypical isolates were considered to be potentially H and grouped according to their morphological similarity.

Extensive sampling. Several cankers in both study plots, from which suspect H isolates had been obtained, were selected for extensive sampling. This included cankers that yielded isolates from each suspect H morphology group and only those not associated with wounded areas or other cankers. Bark plug samples (20 to 24) were removed at 2 to 3 cm intervals near the canker margin and toward the canker center if bark tissue was present. Each plug and the resulting isolates were numbered according to their position in a canker so that the distribution of morphology types could be determined.

Conversion tests. One isolate from each suspect H morphology group was tested for its ability to alter the morphology (convert) of V strains when paired in *vitro* (Anagnostakis and Day 1979). Each type was paired against V strains from 34 different vegetative compatibility (v-c) groups (MacDonald unpublished data) on amended Difco PDA medium at 25 C in a 16-hr photoperiod for 7 to 10 days. Developing V colonies were observed for evidence of conversion. Mycelial transfers were made from V strains that displayed morphological changes to amended PDA medium and grown at 25 C in a 16-hr photoperiod to confirm conversion.

DsRNA analysis. Suspect hypovirulent isolates that were used in the conversion tests and a known V strain were screened for the presence of double-stranded ribonucleic acid (dsRNA). The V strain was included as a control for the extraction process because dsRNA has not been reported in V strains (Day et al. 1977). Mycelia for extraction were grown in six 1000 ml Erlenmeyer flasks containing 125 ml GYE liquid medium at 25 C for 10 days. The mycelial contents from all six flasks of a single isolate were combined and press-dried between layers of absorbent towels. Mycelial pad weights ranged between 3.2 to 5.5 g. Nucleic acids were extracted by methods previously described (Morris and Dodds 1979) with the exception that mycelium was frozen in liquid nitrogen and pulverized with a cold mortar and pestal. The dsRNA was purified according to the procedure described in method 1 of Morris and Dodds (1979) with the addition of a second CF-11 chromatography step. Extracts containing dsRNA were dissolved in 50 u1 electrophoresis buffer (0.09 m Tris, 0.09 M Boric Acid, 0.001 EDTA, pH 8.3) containing 10 percent sucrose, electrophoresed on 5 percent polyacrylamide gels for 10 hr at 10 v/cm and stained with ethidium bromide (5 ug/ml). Ribonuclease digestions were conducted to verify the presence of dsRNA (Morris and Dodds 1979).

Results

During the two-to-three months following inoculation, the host formed callus in the wounded areas and in adjacent portions of the stem that were covered with plastic wrap. In some cases this caused the entire wrapped area to appear swollen. This did not occur in other areas where the bark was unwounded.

Growth of *E. parasitica* from the inoculation sites was indicated by the expansion of necrotic areas in the bark. This was primarily observed at wound sites inoculated with the Italian strains (Table 1) and American strains 209 and 206. However, sustained fungal growth was not usually observed. Limited numbers of pycnidial stromata were formed in these same areas during the first growing season, but usually within a relatively small portion of an inoculation site.

Canker survey and isolate designations. Between May, 1979 and November, 1981, 475 naturally occurring cankers developed in the uninoculated bark tissue of study trees at both locations. Canker incidence was greatest (70) for trees inoculated with strain A-1 and least (19) on trees that received 206 inoculum. Trees inoculated with other H strains had comparable numbers of infections (Figure 1).

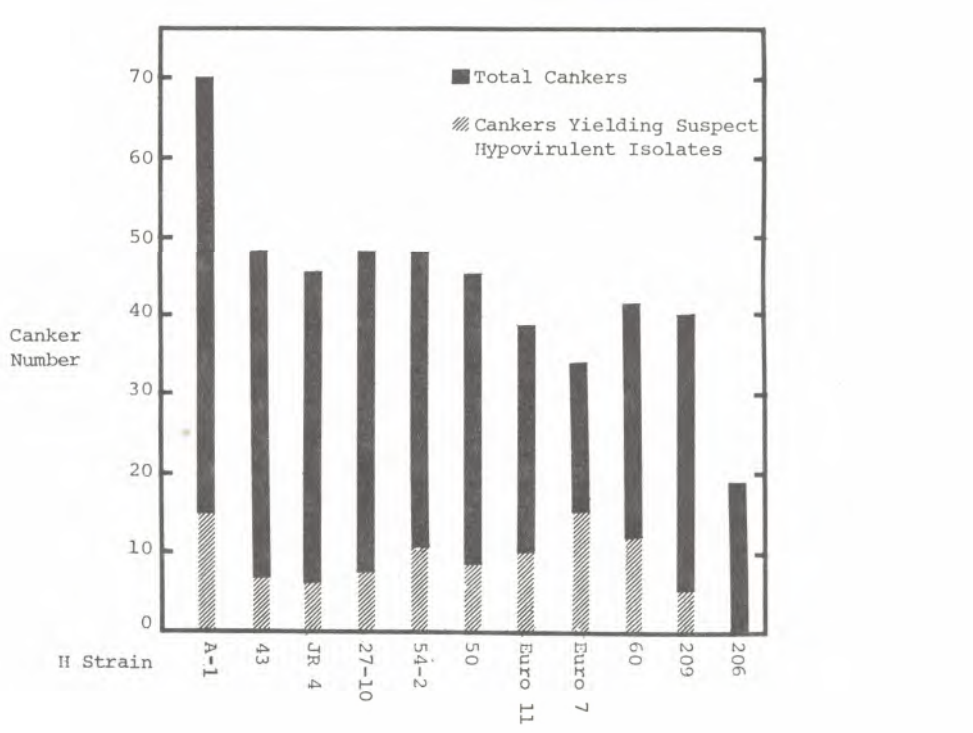

Figure 1. Incidence of naturally occurring cankers and number yielding suspect hypovirulent isolates on American chestnuts inoculated with hypovirulent strains of *Endothia parasitica.*

Eighty-one percent (386) of the naturally occurring cankers yielded only colonies that resembled wild-type V strains. Isolates from the remaining 19 percent (89) displayed atypical morphologies which were considered to represent suspect H types. Suspect H isolates were recovered from cankers on several replicate trees for each inoculum type except 206 (Figure 1). All suspect H isolates from a tree did not always morphologically resemble the H strain that had been inoculated on the tree.

Six distinct suspect H morphology groups were established based on the similarity of isolates in culture (Table 2). Isolates classified in group 1 were recovered most frequently (54 percent) from cankers that yielded suspect H isolates. Group 1 and 2 isolates resembled inoculated H strains 27-10, 54-2, 50, Euro 11, and Euro 7 while group 3 resembled strains 43 and A-1. Group 5 was similar to strain JR 4 except for the presence of curved aggregates of hyphae within the colony. Groups 4 and 6 were not morphologically similar to any of the inoculated H strains.

Table 2. Morphology and frequency of suspect hypovirulent *Endothia parasitica* isolates obtained from naturally occurring cankers

Suspect hypovirulent group	Cultural morphology	Frequency	
1	radially symmetrical growth; white mycelium; yellow-pigmented colony center	48[a/]	(54)[b/]
2	radially symmetrical growth; white	5	(5)
3	slow, erratic growth; white mycelium	5	(5)
4	slow, erratic growth; orange-pigmented mycelium	2	(2)
5	radially symmetrical growth; orange-pigmented mycelium; aggregates of curved hyphae within the colony	8	(9)
6	erratic growth; orange-pigmented mycelium	21	(24)

a/ Total cankers that yielded isolates resembling the suspect hypovirulent morphology group

12/ Percent of all cankers (89) that yielded suspect hypovirulent isolates

Extensive sampling. Twenty-three cankers, from which isolates in groups 1, 2, 3, 5 and 6 had been obtained, were extensively sampled within 20 to 24 months after they were first detected (Table 3). Group 4 cankers were not

Table 3. Morphology groups of *Endothia parasitica* isolates obtained from extensively sampled cankers on American chestnut

Canker	Initial isolate morphology groups	Extensive sample groups	Canker	Initial isolate morphology groups	Extensive sample groups
6-3P[a]	5[b]	6[b] (8)[d] wt[c] (4)	56-1P	3	1 (13) 2 (2)
8-3P	6	6 (11) wt (4)	69-1P	6	6 (7) wt (9)
10-1P	2	2 (10) 3 (1) wt (5)	72-1P	2	6 (3) wt (4)
			89-1P	1	1 (3)
16-2P	1	6 (12) wt (2)			3 (10) wt (1)
26-1P	1	1 (6) 2 (2) 3 (4) wt (1)	100-3P	1	1 (12) 2 (1) 3 (5) wt (1)
34-1P	1	1 (5) 3 (1) 6 (2) wt (8)	1-1B	1	1 (16) wt (1)
			10-3B	1	1 (7) wt (11)
37-3P	1	1 (10) 2 (2) 3 (8) 4 (1) wt (1)	17-1B	1	wt (22)
			49-2B	5	1 (2) 5 (15) wt (2)
37-4P	1	1 (15) wt (2)	75-1B	1	1 (17) wt (1)
42-1P	1	1 (22)			
			87-2B	1	1 (12)
42-2P	1	1 (16) 4 (2) wt (1)			3 (9)
50-1P	1	1 (14) wt (2)	95-2B	1	1 (15) wt (1)

[a] Letters indicate Parsons or Bartow study plot locations.
[b] Number corresponds to suspect hypovirulent morphology group listed in Table 2.
[c] Morphology designation for isolates resembling wild-type virulent strains of *E. parasitica*.
[d] Number in parenthesis indicates total number of samples that yielded each morphology group.

sampled because they had coalesced with other infections by the time extensive sampling was conducted. Group 1 cankers were predominately selected because more were available that had not coalesced with other infections.

Results of the sampling showed that isolates resembling several suspect H morphology groups and wild-type V strains could be present in the same canker (Table 3). From most group 1 cankers, isolates with group 1 morphology were recovered except for cankers 89-1P and 10-3B where other morphology groups predominated. Group 2, 3, 5 and 6 cankers gave fewer isolates that resembled the initial type although isolates from group 5 canker 49-2B predominately resembled the initial group 5 isolate (Table 3). Reconstruction of the cankers based on isolate morphology showed that specific morphology types usually occurred in several areas of a canker as illustrated by canker 37-3P (Figure 2).

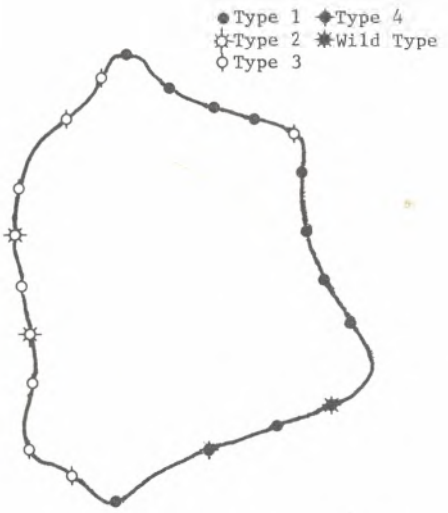

Figure 2. Distribution of *Endothia parasitica* morphology types in naturally occurring canker 37-3P.

Conversion tests. A representative isolate from each suspect H morphology group (Table 2) was able to convert V strains from 3 to 5 different v-c groups (Table 4). In all cases, the converted V strain morphologically resembled the suspect H isolate with which it was paired.

DsRNA analysis. The six suspect H isolates used in the conversion tests were tested for the presence of dsRNA. The dsRNA patterns observed from these isolates and a V strain after ribonuclease treatment in 0.3 M NaCl are shown in Figure 3. Banding patterns before and after ribonuclease treatment were the same for gels 1, 2, 3 and 4. Gels 5 and 6 each contained a single band following electrophoresis that disappeared after ribonuclease treatment in 0.3 M NaCl. All fluorescence disappeared from gels 1, 2, 3 and 4 after incubation with ribonuclease in water verifying the presence of dsRNA.

The isolates from suspect H groups 1, 2, and 3 (Table 2) contained both major and minor segments of dsRNA while the type 4 isolate contained a single major segment (Figure 3). Molecular weights of the segments were not estimated.

Table 4. Virulent strain campatibility groups of *Endothia parasitica* that were converted by suspect hypovirulent isolates in six morphology groups

Isolate	Suspect hypovirulent morphology group	Virulent compatibility groups converted
37-2P	1	A[a/], Y, Z, Delta
99-2P	2	E, Delta, Gamma
5-1P	3	A, E, Y, Z, Gamma
91-1P	4	F, J, Q, Alpha, Iota
64-2P	5	L, Delta, Gamma
10-4P	6	Y, Epsilon, Gamma

[a/] Indicates different compatibility groups (MacDonald unpublished data)

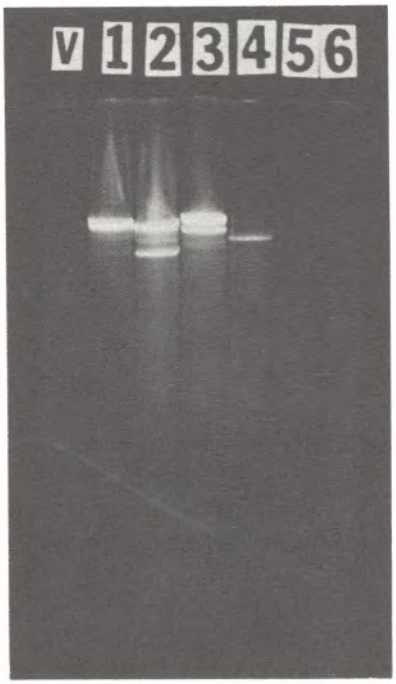

Figure 3. Polyacrylamide electrophoresis gels showing dsRNA banding patterns from suspect hypovirulent morphology groups 1-6; Gel on the extreme left is from a wild-type virulent strain.

Discussion

The majority of the suspect H isolates displayed morphological characteristics similar to those previously described for H strains (Van Alfen et al. 1975; Grente and Berthelay-Sauret 1978; Elliston 1978; 1982). The ability of isolates from each suspect H group to convert the morphology of several V strains is consistent with the *in vitro* behavior of known H strains (Anagnostakis and Day 1979). However, the failure to detect dsRNA in group 5 and 6 is inconsistent with the findings from other H strains (Day et al. 1977). Previous dsRNA extractions have indicated that dsRNA recovery from American H strains is variable (Willey 1980; Elliston personal communication). This could be related to a lower amount of dsRNA (Dodds 1980) and may explain the inability to recover dsRNA from the group 5 and 6 isolates. Extracting from larger amounts of mycelium might enhance the recovery of dsRNA (Elliston personal communication).

At present, results from the morphology evaluations, conversion tests and dsRNA analyses indicate that suspect H isolates in groups 1, 2, 3 and 4 are H. The morphological similarity of groups 1, 2 and 3 to several of the inoculated H strains and their isolation from subsequent naturally occurring cankers suggests that isolates in these groups originated from the H strain inoculation sites. The lack of resemblance between group 4 isolates and any of the inoculated H strains is yet unexplained. However, their origin must also be related to the H strain inoculation sites because morphologically atypical isolates have never been recovered during previous isolations from several hundred cankers in areas of West Virginia where H strains have not been introduced (MacDonald unpublished data).

The occurrence of H isolates in naturally developing cankers is encouraging because it shows for the first time that artificially introduced H strains can disseminate on American chestnuts. The lack of natural dissemination by H strains in previous field control studies (Jaynes and Elliston 1978; MacDonald and Double 1981) may have been due to the use of individual or mixtures of H strains that exhibited low pathogenic and sporulation capabilities (Elliston 1978; Jaynes and Elliston 1980). However, these studies did not report any attempts to obtain *E. parasitica* isolates from cankers that developed following the introduction of H strains. The presence of H isolates in the present study could only be determined by obtaining *E. parasitica* isolates and morphologically evaluating them. This suggests that dissemination of H strains and recognition of hypovirulence in cankers may initially only be verified by sampling.

The incidence of naturally occurring H isolates in this study must be regarded as a minimum number that exists in the study areas. The initial screening of cankers by sampling at only five points may not have been sufficient to detect H isolates in all cankers. Cankers that failed to yield atypical isolates were considered to be V. Yet, the extensive sampling of cankers that contained H isolates showed that both H and V isolates could be present in a single canker. Resampling the V cankers might lead to the recovery of additional H isolates.

The inoculation procedure did not establish abnormal cankers like those found in Italy or Michigan. Canker development of this type may only result from a long-term interaction of H strains with V strains and the host

(Elliston 1982). However, the procedure was successful in establishing H strains and may have helped to promote their natural dissemination. The presence of a disseminating H population has not yet led to noticeable changes in disease incidence on the study trees as V strains continue to cause significant mortality. If the transition of V to H in the *E. parasitica* population is to occur, it may require more than one generation of chestnut sprouts before H strains predominate.

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