THE ABILITY OF HYPOVIRULENT ISOLATES AND MIXTURES OF HYPOVIRULENT ISOLATES TO CONTROL ARTIFICIALLY ESTABLISHED VIRULENT CANKERS

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ABSTRACT.--The *ability of hypovirulent (H) isolates of Endothia parasitica to* alter *the normal morphology of virulent (V) isolates was determined by individually pairing six H isolates and four H mixtures with V isolates from eight vegetative compatibility groups on agar media. The H isolates and H mixtures were scored as either compatible with a V isolate if morphological changes occurred, or incompatible if there was no change. The same V-H combinations (compatible and incompatible) were tested on American chestnut trees to determine whether the in vitro ability of an H isolate or H mixture to alter the morphology of a V isolate was similar to the in vivo ability to control canker expansion. With one exception, V isolates that were compatible in vitro with an H isolate or H mixture were always controlled in vivo. In all cases, H isolates and H mixtures controlled more V isolates in vivo than they morphologically altered in vitro.*

Introduction

The inability of vegetative hyphae to fuse has been reported for many ascomycetes, a few imperfect fungi, and for the chestnut blight fungus, *Endothia parasitica* (Anagnostakis 1978; Andes 1961; Caten 1972). When hyphae of two colonies of *E. parasitica* fail to anastomose on agar media, the result is the formation of a barrage, consisting of a ridge of asexual fruiting structures (Anagnostakis 1978; 1981b). This reaction has been termed vegetative incompatibility, and occurs commonly in North America among virulent (V) strains of *E, parasitica* (Anagnostakis 1981a; MacDonald and Double 1978). Genetic controls apparently govern the hyphal fusion and thereby determine vegetative compatibility (v-c) type (Anagnostakis 1981b; Day 1978). The restriction of fusion, due to vegetative incompatibility, may interfere with the transmission of hypovirulence determinants necessary for the conversion of a V strain to a hypovirulent (H) form (Anagnostakis 1978; Anagnostakis and Day 1979; Grente and Berthelay-Sauret 1978). The conversion of V strains to the H form occurs easily *in vitro* between strains of the same v-c type, but if the two strains are not of the same v-c type, conversion is sometimes slow and erratic, or may not occur (Anagnostakis and Day 1979).

The purpose of this study was to determine if V isolates, whether compatible

or incompatible in *vitro* with H isolates and H mixtures, were controlled in *vivo* when challenged with the same H isolates and mixtures.

Materials and Methods

The individual H isolates and the components of the H mixtures used in this study are listed in Table 1. Isolates were selected for each mixture, except the general, on the basis of their morphologic and pathogenic similarities (i.e. all components of the B mixture were white and nonpathogenic). The

Table 1. Components of the four hypovirulent mixtures

Designation	Components
B (nonpathogenic, white)	EP-14[a], EP-43[a], Grente's HV[b] 1-4-2w[c], 4-9-2w[c], 4-10-1w[c]
JR (nonpathogenic, pigmented)	JR 1[a], JR 2[a], JR 4[a], 4b7[a]
Italian (moderate pathogenicity and pigmentation)	EP-47[a], EP-48[a], EP-49[a], EP-50[a] EP-51[a]
General (varying pathogenicity and pigmentation)	All isolates listed above, plus EP-60[a], EP-90[a], EP-93[a], EP-102[a]

[a] Obtained from the Connecticut Agricultural Experiment Station, New Haven, CT 06504.

[b] Obtained from J. Grente, Clermont-Ferrand, France.

[c] Obtained from reisolations from natural cankers artificially inoculated with EP-43, West Virginia.

general mixture was comprised of all isolates in the B, JR, and Italian mixtures, plus four American components, EP-60, EP-90, EP-93, and EP-102. Inocula were prepared by first growing each of the 19 H isolates in three 250 ml Erlenmyer flasks containing 50 to 75 ml of semi-solid glucose yeast extract (GYE) agar, which consisted of 10.0 g of glucose, 2.0 g of Difco Bacto yeast extract, 1.0 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.2 mg of Fe^{++}, 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 1,000 ml of distilled water (Lilly and Barnett 1951). The flasks were then incubated at 25 C in a 16-hr photoperiod for 8 to 10 days and each flask was shaken vigorously after 3 days to enhance growth.

All 18 flasks of the six components comprising the B mixture were combined in a Waring blender and mixed at high speed for 45 to 60 seconds. Sterile solidified water agar (20 g/l) was added to 500 ml of this semi-solid mixture and blended until a loose gel consistency was obtained. The blended mixture was then transferred to 250 ml wash bottles for field use. The JR and Italian mixtures were prepared similarly, by combining their respective components.

The general mixture was prepared by combining equal amounts (approx. 100 ml) of the semi-solid B, JR, and Italian mixtures. The four American H components

were added to this mixture which was also thickened as above.

In vitro studies. Six H isolates and four H mixtures (Table 2) were paired separately against eight V isolates of different v-c types (A, B, C, D, E, F, G, and N) by the method described by Anagnostakis (1977; 1978). All tests were conducted by pairing mycelium plugs (2 to 3 mm square) of one V and one

Table 2. Virulent v-c types of *Endothia parasitica* that are converted *in vitro* and in *vivo* by individual hypovirulent (H) isolates and mixtures of H isolates

	v-c type converted		
		In vivo test	
Type	*In vitro* test[a/]	Controlled	Not controlled
Individual H Isolates			
EP-49	N	C, F, N	A, B
EP-50	N	C, F, N	A, B
JR 1	None	C, F, N	A, B
JR 4	N	C, F, N	A, B
1-4-2w	A	A, C, F	B, N
4-10-1w	B, F	C, F, N	A, B
H Mixtures			
B	A, B, D, E, F	A, B, C, D, E, F, G, N	-
JR	N	C, F, G, N	A, B, D, E
Italian	N	C, D, E, G, N	A, B, F
General	A, B, C, D, E, F, N	A, B, C, D, E, F, G, N	-

a/ Individual H isolates were tested *in vitro* against v-c types A, B, C, F, and N while the H mixtures were tested against v-c types A, B, C D, E, F, G, N.

H isolate on PDAmb (Difco potato dextrose agar supplemented with 0.1 mg/1 of methionine and 0.01 mg/1 of biotin) and incubated at 25 C in a 16-hr photoperiod (white fluorescent light) for 10 days (Anagnostakis 1977). Only one pair was tested per petri plate. Tests involving the mixtures were similar, however, in place of the H mycelium plug, a 0.5-cm diameter disk of agar was aseptically removed adjacent to the V plug and the resulting well was filled with the appropriate H mixture. All H and V combinations were replicated at least twice. Virulent isolates were scored as converted when a change in their morphology was observed (Anagnostakis and Day 1979).

In vivo studies. Virulent cankers of *E. parasitica* were artificially established on 39 American chestnut trees *(Castanea dentata)* in July 1978, for eventual challenge with H mixtures. Eight inoculations per stem, 15 to 20 cm apart, were made by removing a 0.5-cm diameter bark plug with a cork borer. A different V isolate from each v-c type (A, B, C, D, E, F, G, N) was inoculated into each wound by placing a similar size piece of agar containing V

mycelium into the inoculation site. These sites were covered with masking tape to retard drying. The V cankers were challenged 4 to 5 weeks after their initiation by punching 0.5-cm diameter bark plugs every 1 to 2 cm around the canker margin. The resulting holes were then filled with one of the four H mixtures and covered with masking tape. A similar treatment but with water agar was used as a check.

Twenty-seven additional American chestnut trees were inoculated with V isolates representing v-c types A, B, C, F, and N, for eventual challenge with H isolates. Each V isolate was introduced at two sites per stem. After 4 to 5 weeks, the resulting cankers were challenged with H isolates 1-4-2w, 4-10-1w; JR 1, JR 4; EP-49, and EP-50, selected from the B, JR, and Italian mixtures, respectively. These particular isolates were chosen according to their ability to convert certain v-c types *in vitro*. The V cankers were challenged as above, except an agar plug containing H mycelium was used in place of the H mixture. The length and width of each canker were measured at the time of the H challenge 3, 9, 15, 20, and 27 months after the challenge, and measurements were recorded either as identical in size at the time of the challenge or larger. Data are presented (Tables 3 and 4) as an average linear growth (cm) of each canker after 27 months and were determined using the formula $\frac{(L + W)}{2}$.

Table 3. Average linear growth (cm) of virulent v-c types of *Endothia parasitica* 27 months after challenged *in vivo* with four hypovirulent mixtures and water agar.

V-c type	Hypovirulent Mixtures y/				Water agar
	B	JR	Italian	General	
A	5.42b	10.89a	9.42a	4.25b	11.06a
B	4.75b	8.75a	10.42a	4.83b	9.67a
C	11.19b	11.92b	12.08b	5.83c	17.83a
D	5.25c	13.69a	9.19b	5.86c	15.33a
E	5.81c	10.14ab	8.67bc	5.92c	13.33a
F	6.31b	7.94b	11.92a	7.78b	15.33a
G	6.28c	9.17bc	12.31b	8.28c	19.83a
N	9.44b	6.75b	6.17b	8.00b	15.00a

y/ Average values followed by the same letter in each row do not differ significantly at P = 0.05.

Vegetative compatibility types were listed as either controlled, when the average linear growth of the challenged canker was significantly smaller than the water agar check, or not controlled when the significance values of the challenged canker and the check did not differ (Tables 3 and 4).

Results

Results of both the laboratory conversion *(in vitro)* test and the field challenge *(in vivo)* are in Table 2. The H mixtures were no better than the individual components comprising the mixtures when conversion tests with V

Table 4. Average linear growth (cm) of virulent v-c types of *Endothia parasitica* 27 months after challenged *in vivo* with six individual hypovirulent isolates and water agar

V-c type	Individual Hypovirulent Isolates[y]						Water agar
	EP-49	EP-50	JR 1	JR 4	1-4-2w	4-10-1w	
A	10.50ab	7.78bc	7.11bc	9.47abc	7.58bc	9.61abc	11.06a
B	14.72a	8.52b	8.25b	6.72b	7.97b	8.08b	9.67b
C	13.06b	10.86bc	11.05bc	10.72bc	7.97c	10.06bc	17.83a
F	11.89b	9.08bc	6.70c	7.08c	6.52c	6.72c	15.33a
N	9.61b	5.94c	9.05bc	6.02c	14.08a	8.38bc	15.00a

[y] Average values followed by the same letter in each row do not differ significantly at P = 0.05.

isolates of different v-c types were conducted in *vitro.* However, when the same V isolates were challenged in the field test, both the H mixtures and H isolates controlled more cankers than either did *in vitro* or example, the Italian mixture, and two of the Italian components, EP-49 and EP-50, only converted v-c type N in *vitro.* Yet, the same mixture controlled the expansion of cankers incited by v-c types C, D, E, G, and N in the field test, and EP-49 and EP-50 controlled v-c types C, F, and N (Table 2). With one exception, all V isolates which were converted from the V to the H form *in vitro,* were also controlled in the field test by both individual H isolates and H mixtures. The exception was H isolate 4-10-1w which converted v-c type B in the laboratory, but did not control the canker expansion of v-c type B in the field.

Treatment with either the B or general mixtures resulted in significantly smaller cankers for each of the eight v-c types, when compared to cankers treated with water agar (Table 3). The JR and Italian mixtures only controlled canker expansion of four and five v-c types, respectively. No individual H isolate controlled canker expansion of all v-c types, but each of the individual H isolates was consistently better in *vivo* than *in vitro.*

Discussion

The chestnut blight fungus, *E. parasitica,* is commonly disseminated by ascospores (Anderson 1913; Anderson and Babcock 1913; Heald et al. 1915). Perithecia from a single wild-type canker frequently produce ascospores that are of different v-c type (Anagnostakis 1977). Consequently, ascospores that are infectious presumably would produce cankers that differ in v-c type. This seems to be the case for areas in North America where the v-c types of cankers have been determined (Anagnostakis 1981a; MacDonald and Double 1978). The diversity of v-c types found in the forest may significantly limit the transfer of cytoplasmic agents if the V and H strains do not interact because they are vegetatively incompatible. The findings of this study are encouraging because some v-c types that were not converted on agar media were controlled in the field test. For example, the B mixture converted v-c types A, B, D, E, and F in the laboratory, but controlled cankers incited by all v-c types challenged in the field.

Several reasons may exist for the more successful interaction of V and H isolates *in vivo.* One explanation may lie with the increased potential for anastomosis between V and H isolates afforded by the tree. The pattern of growth of *E. parasitica* within bark may provide greater opportunity for V and H mycelium to anastomose over a longer period of time than achieved in culture. Another explanation may involve the genetic controls that govern vegetative compatibility in *E. parasitica.* In an experiment where V and H strains were coinoculated in American chestnut trees, canker expansion was significantly limited when v-c types of the two strains differed at 0, 1, or 2 loci, but not significantly limited when the two strains differed at 5 or more loci (Anagnostakis and Waggoner 1981). Although the genetic relatedness among the V and H isolates used in this study is not known, the same genetic controls may be responsible for the successful in *vivo* control of combinations that were incompatible *in vitro.*

The term "control" was used in this study when the size increase of cankers treated with H inoculum was significantly less than cankers treated with water agar. The statistical tests undoubtedly were influenced by the method of canker measurement. In all cases, canker measurements were recorded either as identical to treatment size or larger. However, formation of callus sometimes resulted in the reduction in size of some cankers after treatment. If reduction in canker size had been considered, greater differences between the H and water agar treatments would have resulted, and the one V-H combination, v-c type B and H isolate 4-10-1w, that was successful in the laboratory may also have been successful in the field.

An additional problem was encountered with the virulent v-c type B isolate. The v-c type B cankers treated with EP-49 were significantly larger than the water agar checks. The increased canker size may not be due to the failure of EP-49 to control this particular v-c type, but might reflect the growth potential of this H isolate. Other workers have shown that EP-49 can colonize host tissue as readily as normal strains (Elliston 1978; Willey 1980). Table 4 illustrates that EP-49 treated cankers were almost always the largest of the H-treated cankers. Despite its high growth rate, EP-49 controlled as many cankers as the other five H isolates.

Data from this experiment has shown that individual H isolates and H mixtures more effectively control V isolates in a field situation, than suggested by their interaction *in vitro.* Vegetative compatibility may therefore not be as great a barrier to success of H as first thought. At this point, other factors such as method of establishing H inoculum, would seem to be a greater obstacle to success of H as a biological control.

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