Interactions Between Artificially Established Virulent *Cryphonectria parasitica* Cankers and Sources of Virulent and Hypovirulent Inoculum on American Chestnut Stems

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ABSTRACT. The success of hypovirulent (hv) strains, as biological control agents for chestnut blight in forest settings, depends on their effective dissemination and interaction with virulent (v) strains. This study assessed the role of vegetative compatibility by examining the interactions between vegetatively compatible and incompatible v and hv strains of *Cryphonectria parasitica* artificially inoculated on American chestnut stems. Color mutants, a pcnb tolerant strain, and a morphologically distinct hv isolate were used so that interactions could be measured. Cankers were established on stems with a brown-pigmented strain of *C. parasitica*, allowed to develop for 8 wk, and then exposed for 10, 20 or 52 wk to vegetatively compatible or incompatible orange-pigmented v or hv inoculum. Laboratory inoculated bark patches, placed against the bark 10 cm above the cankers, served as inoculum sources. Cankers were harvested and bark plugs removed and cultured. Resulting isolates were assessed for pigmentation, morphology and sensitivity to pcnb. Final canker dimensions also were evaluated.

Seven different types of *C. parasitica* isolates were recovered from the cankers. The brown v strains, used for canker initiation, were recovered most commonly from the treatments. The exception was the hypovirulent-compatible treatment (brown v thallus exposed to compatible hv inoculum) in which 56%, 82% and 95% of the isolates from the 10-, 20- and 52-wk exposure periods, respectively, were brown-hv. Cankers exposed to vegetatively incompatible hv inoculum yielded 5%, 1% and 31% brown-hv isolates from their respective exposure periods. Canker growth was not controlled in any treatment. Results indicate that vegetative compatibility is a primary barrier to canker conversion. Once compatibility is overcome rapid conversion occurs. Other interaction products were recovered in isolation. These products indicate that several strains may inhabit cankered bark.

Strains of *Cryphonectria parasitica* (Murr.) Barr, associated with surviving trees in Italy, were shown to be less virulent than strains that had previously been lethal to European chestnut (5, 14). These strains, termed hypovirulent (hv) because of their reduced virulence, were obtained by American scientists to determine whether the European hv isolates could be utilized for biological control of the disease on American chestnut *(Castanea dentata* [Marsh.] Borkh.) (15). To date, research has determined that hv strains can be used effectively to control v cankers when preparations of conidia and/or mycelium of hv strains are applied directly to a canker (3, 7, 9, 13). However, the success of hv isolates in providing natural control of chestnut blight may depend on their ability to disseminate and interact effectively with the population of v isolates. The intent of this research was to examine the dissemination and interaction of v and hv isolates *in* a forest setting.

MATERIALS AND METHODS

Sixty American chestnut sprouts growing in the Pernow Experimental Forest near Parsons, W.Va. were selected for this study. The site, selectively cut in 1977, resulted in a partial canopy that allowed for release of understory chestnut. Study trees ranged from 3.0 to 9.5cm (avg. 4.9) cm in diameter, 1.4 m above the ground.

Four strains of *C. parasitica* were used for the experiment. The first strain, 5-9-1B, is a v isolate from West Virginia that is pigmented brown and is in vegetative compatibility (v-c) group A. This isolate was designed Brown A. A second brown v isolate, in v-c group B, was designed Brown B. These two strains were used to establish four cankers on each of 60 trees. Cankers on 24 trees were initiated with Brown A, and cankers on the remaining 36 trees were initiated with Brown B; 12 of these served as controls. Cankers were established at 0.5, 1.0, 1.5 and 2.0 m above the ground, one in each cardinal direction. Inoculation was accomplished by removing a bark plug with a cork borer and inserting a similar size mycelial plug of the appropriate brown isolate. The inoculation sites were covered with masking tape to reduce dessication, and cankers were allowed to develop for 8 wk before exposure to test inocula.

Two isolates were used to provide sources of v and hv inoculum to interact with the brown-initiated cankers, Euro-7 and CL1-BLBO. Euro-7 is a lightly pigmented orange hv strain of Italian origin. CL1-BLBO is a bright orange v isolate from Michigan that is tolerant to the fungicide pentachloronitrobenzene (pcnb). Both isolates are in v-c group A.

The inoculum sources were generated by culturing Euro-7 and CL1-BLBO on American chestnut bark

patches in the laboratory. Bark, peeled from healthy chestnut stems and cut into 5 x 8 cm rectangles, served as substrates for the artificial inoculum. After being scratched lightly with a dissecting needle 10-12 times per side to enhance sporulation, the patches were placed on end in 160 mm diameter desiccator jars. One hundred ml of liquid glucose-yeast extract (GYE) medium were added to each desiccator. The desiccators were covered with aluminum foil and autoclaved for 30 min at 121 C, 15 lb/in^2 pressure. The bark patches were inoculated with agar plugs of Euro-7 or CL1-BLBO and incubated at 25 C for 4-6 wk (16-h photoperiod), or until adequate bark colonization and sporulation were apparent. Eight wk after cankers were intitiated, bark patches were mounted on the study trees 10 cm above the leading edge of each canker (Figure 1a), with the cambium side placed toward the stem and attached with thumb tacks at each corner. The inoculum patches were replaced monthly during the growing season. Uninoculated sterile bark patches served as controls on 12 trees.

Treatments. The treatment combinations consisted of exposure of the v cankers, initiated by Brown-A or Brown-B, to bark patches with sporulating Euro-7 (hv) or CL1-BLBO (v), or to uninoculated sterile bark patches (Table 1). The combinations resulted in the v cankers being exposed to v or hv, vegetatively compatible or incompatible inoculum. Each treatment consisted of 4 trees with all 4 cankers on each tree exposed to the same inoculum. Sets of cankers in each treatment were exposed to inoculum sources for 10, 20 or 52 wk.

Table 1. Combinations of canker and inoculum sources.

Patch*	Treatment Type	Canker	Inoculum
HC	Hypovirulent Compatible	Brown-A	Euro-7
HI	Hypovirulent Incompatible	Brown-B	Euro-7
VC	Virulent Compatible	Brown-A	CL1-BLBO
VI	Virulent Incompatible	Brown-B	CL1-BLBO
C	Control	Brown-B	Sterile Bark Patch

*Both orange inoculum isolates, Euro-7 and CL1-BLBO, are vegetatively compatible with Brown-A.

Laboratory Procedures. At the end of each exposure period, trees were harvested in the field and the cankered sections were cut from each stem. Each canker was labeled, placed in two plastic bags to prevent desiccation, and frozen at −10 C. Cankers were removed from the freezer and sampled as time permitted. Each cankered section was measured for canker length and width. Cankers were oriented right-side up and bark plugs removed using a #2 cork-borer. Twelve bark plugs were removed from the canker face (Figure 1b); eight from the canker

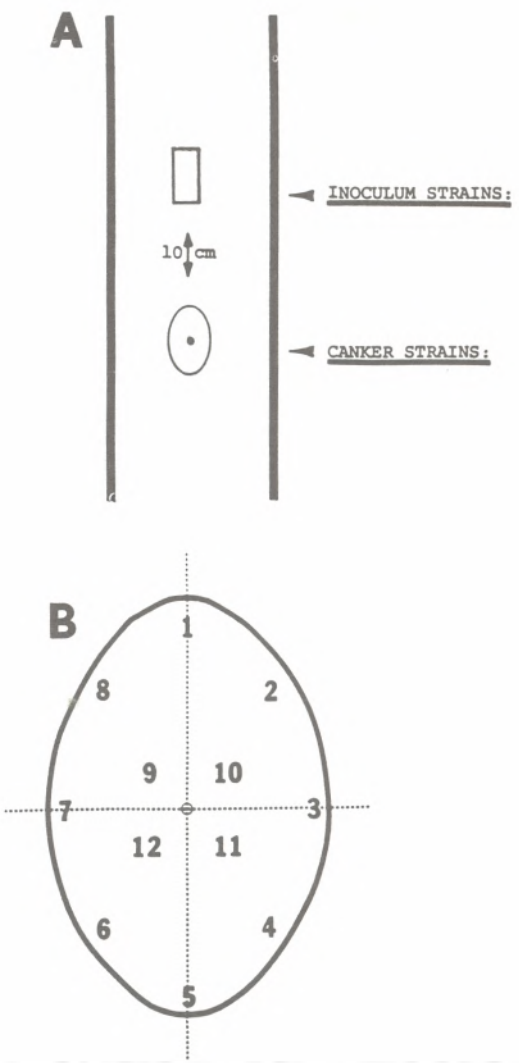

Figure 1. a. Bark patch inoculum source mounted 10 cm above leading edge of v canker. b. Location of canker sampling positions.

margin and four from the interior. Plugs were surface sterilized for 10 min in a 0.5% sodium hypochlorite:0.1% Tween-20 solution followed by two immediate rinses in sterile distilled water. After sterilization the bark plugs (four per Petri plate) were transferred onto GYE medium amended with 3 mg/I streptomycin sulfate and 50 mg/1 chlortetracycline. After 2-3 days incubation at room temperature, developing mycelia were transferred onto a potato dextrose agar medium amended with 1 mg/1 methionine and 5 g/1 biotin (PDAmb, Difco, Detroit, Mich.). Cultures were evaluated after 7-10 days incubation at 25 C with a 16-h photoperiod. The identity of each isolate was maintained through the entire sampling and culturing process using a system that incorporated tree number, canker height and isolate position within the canker (e.g. 4-2.0-12).

Isolate Evaluation. Isolates were categorized initially based on morphology, pigmentation, tolerance to pcnb and v-c type. All orange-pigmented isolates morphologically similar to CL1-BLBO were evaluated for their toler-

ante to pcnb to determine whether they originated from the CL1-BLBO inoculum strain. To do this, each isolate was tested and compared with Euro-7 and CL1-BLBO on PDAmb amended with 100 mg/1 of pcnb of Terra Coat L 205, Olin Chemical liquid pcnb (UniRoyal Chemical Company, Detroit, Mich.).

Vegetative compatibility was tested, using the methods of Anagnostakis (1), for all orange isolates that appeared morphologically similar to CL1-BLBO that were not tolerant to pcnb. Orange isolates that were vegetatively compatible with the v isolate derived from Euro-7, were classified as Euro-7 virulents. Orange isolates not vegetatively compatible with the known Euro-7 v were categorized as wild types. Virulent brown isolates were tested for v-c against the original brown strains used to establish the cankers. Selected isolates were tested for the presence of dsRNA using a modification of the procedures employed by Morris and Dodds (11).

Some isolates were recovered that produced brown and orange sectors while others were "blended" as if they represented a blending of brown-orange pigments. The brown and orange portions of the sectored and blended pairs were evaluated individually. The colonies that appeared to be blended brown-orange were further characterized by either single sporing or hyphal-tip culturing.

Single sporing was done by aseptically wetting cultures with 2-3 ml of sterile distilled water. The spore suspension was serially diluted to provide 75-100 spores/ml, and plated onto GYE medium amended with antibiotics. After 25-30 h at room temperature, individual germinating conidia were transferred, three per plate, onto PDAmb. Cultures were incubated for 5-7 days at 25 C with a 16-h photoperiod, and evaluated for their morphology.

Hyphal tip culture was accomplished by selecting eight of the blended isolates and transferring them from PDAmb onto water agar. Several sequential transfers on water agar were necessary until hyphae were sparse enough that single hyphal tips could be picked with a needle and transferred onto PDAmb. Hyphal tips were cultured at 25 C for 7-10 days (16-h photoperiod).

The orange component of each sectored or blended isolate was evaluated in the same manner as individual orange or brown isolates.

Statistics. Chi-square was used to analyze the brown and brown-hv isolates recovered from the 12 sample positions on cankers exposed to hypovirulent inoculum (HC and HI treatments). One analysis tested each position (1-12) separately for the effects of treatment, exposure and canker height on the type of isolate recovered. A second chi-square combined the 12 sample positions and analyzed for differences in the type of isolate recovered from one position to the next.

RESULTS

Isolates of *C. parasitica* were routinely recovered from 98% of the bark plug samples taken from test cankers. Pigmentation, colony morphology, tolerance to pcnb and v-c type were useful criteria in separating the isolates into one of seven categories (Table 2).

Table 2. Classification of recovered isolates.

Classification	Description
Brown-Pigmented Isolates	
Brown	Morphologically similar to Brown A or Brown B
Brown hv	Pigmented light-brown, but morphologically similar to Euro-7
Abnormal Brown	Pigmented light-brown, but slow growing
Orange-Pigmented Isolates	
CL1-BLBO	Morphologically similar to CL1-BLBO and pcnb tolerant
Wild Type	Morphologically similar to CL1-BLBO; pcnb intolerant and vegetatively incompatible with a v isolate derived from Euro-7
Euro-7 virulent	Morphologically similar to CL1-BLBO; pcnb intolerant and vegetatively compatible with a v isolate derived from Euro-7
Euro-7	Pigmented light orange, morphologically similar to Euro-7; pcnb intolerant

The isolates most commonly recovered from all cankers were the brown v isolates, (similar to the isolates used to initiate the cankers), accounting for 73% of all isolates recovered. The second most common isolate was the brown-hv, which accounted for 20% of all isolates. This isolate represented an interaction product between the brown isolates used to establish the cankers and the orange hv isolate, Euro-7. Brown-hv isolates occurred primarily in cankers that were exposed to vegetatively compatible hv inoculum (HC treatment, Table 3). In the HC treatment, brown-hv isolates accounted for 56%, 83% and 95% of all isolates recovered from 10-wk, 20-wk and 52-wk exposure periods, respectively. Cankers exposed to vegetatively compatible hv inoculum for only 10-wk showed an effect of canker height, where a significantly greater number of brown hv isolates were recovered from each lower canker (38%, 40%, 69% and 77% from cankers at 2.0, 1.5, 1.0 and 0.5 m, respectively) (data not shown).

In contrast to the HC treatment, cankers exposed to hv incompatible inoculum (HI treatment) yielded only 5%, 1% and 31% brown-hv for 10-wk, 20-wk and 52-wk exposure periods, respectively. Unexpectedly, some virulent cankers, exposed to virulent-compatible inoculum (VC treatment) also yielded brown-hv isolates. This occurred at all three exposure periods, where 2%, 13% and 8% of the isolates recovered were brown hv, respectively. No brown-hv isolates were recovered from cankers exposed to virulent incompatible inoculum (VI treatment). How-

ever, 4% of the isolates from 52 wk control cankers yielded brown-hv isolates (Table 3).

Euro-7 and CL1-BLBO (the bark patch strains), as well as Euro 7-virulent and *C. parasitica* wild-type isolates, were recovered only a few times. Euro-7 was isolated only rarely, and only from cankers exposed to that inoculum (data not shown). Wild-type virulent isolates occurred in low numbers in all treatments (1.7% of all isolates).

A small number of bark plugs (1.6%) yielded isolates that appeared as sectored colonies in culture. These isolates usually consisted of pairs of isolates, primarily a brown and an orange isolate. The most common isolate pair was the brown v and orange wild-type. Isolate pairs were recovered in all treatments.

Chi-square analysis revealed no significant difference in the type of isolate (brown, brown hv or other) recovered from the canker based on position, or treatment and exposure period.

Detection of dsRNA. A representative sample of v and by isolates were tested for the presence of dsRNA. All brown-hv isolates tested yielded one major dsRNA band (- 10-11 kb). Double-stranded RNA was not detected in any of the isolates rated as brown v.

DISCUSSION

Virulent cankers can be converted to hv by direct application of agar plugs, slurries or conidial sprays (3, 7, 9, 13). Further, Garrord, Ravenscroft and Fulbright (4) demonstrated that hv inoculum could be disseminated to create hv cankers on wounded trees. They also noted some conversion of v to by strains. This study demonstrated that by inoculum could be naturally disseminated to a virulent canker and cause its conversion to hv. The use of marked strains permitted identification of isolates and closer study of their interactions.

The role v-c plays appears to be the most significant factor in the conversion process. Exposure of v cankers to hv compatible inoculum results in nearly complete conversion (95% conversion after 1 yr of exposure). Even after exposure for only 10 wk over 112 of isolates recovered from compatible cankers had hv morphology (Table 1). After 20 wk, 83% of isolates from compatible cankers displayed hv morphology. Hv isolates (brown hv) also were recovered from compatible cankers exposed to v inoculum where, after only 20 wk, 13% of isolates were brown-hv. This, however, did not increase with time.

There also is an indication that the concentration or quantity of inoculum may initially increase conversion. In the 10-wk period, lower cankers yielded a greater number of converted isolates than cankers higher on the tree. This may be the result of lower cankers receiving greater amounts of inoculum. This additive effect was not evident in longer exposure periods but this may be a result of the greater conversion that occurred in cankers at all heights.

Conversion of the virulent canker thalli occurred in incompatible treatments but at lower rates. In cankers exposed to hv-incompatible inoculum for 10 or 20 wk, less than 5% of the isolates were hv (Table 1). After a year however, almost 31% of isolates recovered from incompatible cankers had hv morphology. Even though compatibility can be overcome it is the most significant barrier to conversion once inoculum is delivered to the canker. Incompatible cankers exposed to v inoculum showed no conversion. The only other brown hv isolates recovered were from control cankers. These were vegetatively incompatible cankers; however, 4% of the isolates were brown hv, most of these occurring on one tree.

Table 3. Percentage of isolate types recovered from the five treatment combinations.*

Isolate	Exposure Period 10-wk	20-wk	52-wk	Exposure Period 10-wk	20-wk	52-wk	Exposure Period 10-wk	20-wk	52-wk
	Control Treatment			*Hv-Compatible Treatment*			*Hv-Incompatible Treatment*		
Brown	81	99	83	42	15	4	89	98	65
Brown-Hv	–	–	4	56	83	95	5	1	31
Wild-Type	1	1	9	1	–	–	1	0	1
CL1-BLBO	–	–	–	–	–	–	–	–	–
Brown+Orange**	2	–	3	–	–	–	3	1	3
Other†	17	–	1	1	2	1	3	1	1
				V-Compatible Treatment			*V-Incompatible Treatment*		
Brown				84	87	91	84	82	98
Brown-Hv				2	13	8	–	–	–
Wild-Type				1	–	–	–	13	–
CL1-BLBO				3	–	–	7	1	1
Brown+Orange**				4	–	–	3	4	1
Other†				5	–	1	7	–	–

*Approximately 192 bark samples/exposure period
**Sectored and blended isolates
†Contaminants, Euro-7 and blanks

Pycnidia were the primary fructifications produced on inoculum patches. No perithecia were observed. During the first 20 wk of the study at least 6 rains occurred, all of which were 13 mm (0.5 in). This would have been adequate for conidial dissemination by stem flow. In addition, when inoculum patches were replaced, insects (ants and centipedes) were observed under them. Mites, weevils and ants were commonly observed on study trees throughout the growing season. Insects have been shown to be carriers of *C. parasitica* (2, 6, 12); therefore, insects or other animals may have been responsible for the dissemination of hv inoculum to trees where hv isolates were recovered. Garrod, Ravenscroft and Fulbright (4) also noted tree to tree spread of inoculum strains.

No reduction in canker growth was evident as a result of conversion from v to hv. In fact, most, if not all, of the study trees presumably would have been girdled had they not been destructively sampled. This result was not surprising as the hv isolate, Euro-7, is relatively virulent. It was chosen because of its ability to produce inoculum. Reduction in canker growth was not a goal of this study but has been demonstrated by previous studies (8).

Interactions occurred in all treatments between the v canker thalli and naturally introduced strains of *C. parasitica.* In addition to the dominant brown v isolates and the brown-hv interaction product, other isolates also were recovered, although infrequently. Even with the large amount of Euro-7 and CL1-BLBO inoculum present, only a small number of these isolates were recovered. Most of them were recovered from the 10-wk exposure cankers. This leads one to believe that only a limited chance exists for successful colonization of previously cankered bark. If this is true, then the rate of virulent canker conversion is even more stunning.

The most commonly recovered isolates, other than the brown or brown-hv, were wild-types. These accounted for approximately 2% of all isolates recovered. This is not surprising as the study area was surrounded by naturally infected chestnuts. For the most part these isolates occurred randomly except for one tree with 3 cankers where 25 wild-type isolates were recovered. Possibly wild-type strains became established in the bark soon after inoculation, resulting in their domination of those cankers.

Isolate pairs recovered from a single bark plug consisted of a brown isolate (brown-A or -B) growing with an orange isolate (wild-type or CL1-BLBO). Both components of these 8 pairs appeared to be coinhabiting the bark. Recovery of more than one isolate from a bark plug is not unusual, as Kuhlman (10) demonstrated that cankers may contain two or more strains of different v-c types.

The rapid conversion of v cankers by hv inoculum makes a strong case for the potential usefulness of hv strains as biological control agents. Although the limitations imposed by v-c appear significant, studies have demonstrated that vegetative incompatibility in cankers can be overcome (3, 10). This experiment also indicates that, given time and sufficient exposure to hv inoculum, vegetative compatibility may become less of a barrier to conversion.

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