Dissemination of Virulent and Hypovirulent Forms of a Marked Strain of *Endothia parasitica* in Michigan

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Support is gratefully acknowledged from the Northern Nut Growers Association, the Michigan Nut Growers Association, the Michigan State University Development Fund, and Chimney Corners Resort, Frankfort, MI.

Michigan Agricultural Experiment Station Journal Series Article 11429.

Accepted for publication 20 November 1984 (submitted for electronic processing).

ABSTRACT

Garrod, S. W., Fulbright, D. W., and Ravenscroft, A. V. 1985. The dissemination of virulent and hypovirulent forms of a marked strain of *Endothia parasitica* in Michigan. Phytopathology 75:533-538.

The infection and spread of a virulent and a hypovirulent form of a pentachloronitrobenzene-resistant strain of *Endothia parasitica* was examined in an American chestnut (*Castanea dentata*) grove. After electrophoresis in polyacrylamide gels, the hypovirulent form exhibited a unique dsRNA banding pattern that enabled monitoring the spread of specific dsRNA segments. Spread of virulent strains and hypovirulent strains (carrying the specific dsRNA bands) was detected within and among trees. Conversion in situ of virulent strains to hypovirulent strains occurred on some trees. Wounds located 10–110 cm from inoculum sources were infected equally at the end of 15 mo.

Chestnut blight, caused by the fungus *Endothia parasitica* (Murr.) And. and And., was responsible for the demise of the once prevalent American chestnut (*Castanea dentata* [Marsh.] Borkh.) in North America. Today the chestnut blight pathogen continues to kill regrowth from stumps throughout New England and the Appalachian forest (5). In Europe and in certain groves in MI, chestnut trees survive and produce nuts in spite of infection by *E. parasitica* (6,7). We believe the survival of these trees in MI is due to the presence of hypovirulent strains of *E. parasitica* (7). Hypovirulent strains are less virulent than virulent strains and contain double-stranded ribonucleic acid (dsRNA) that can greatly reduce the aggressiveness of virulent strains. Hypovirulent strains have been used to arrest individual cankers initiated by virulent strains (5).

It may be possible to control chestnut blight through the use of hypovirulence, provided there is natural dissemination of hypovirulent strains within groves. Willey (18) reported the spread of hypovirulent *E. parasitica* among cankers on trees in West Virginia that had been inoculated previously with hypovirulent strains. This procedure was successful in establishing hypovirulent strains on the same tree and suggested that hypovirulent strains could spread to other trees (W. L. MacDonald, *personal communication*).

The objective of this research was to establish a hypovirulent form of *E. parasitica* in an American chestnut grove and to compare the spread of the genetically marked hypovirulent strain of *E. parasitica* with that of virulent strains in a blighted American chestnut grove. The hypovirulent strain contained a unique dsRNA banding pattern observable after electrophoresis in polyacrylamide gels and, therefore, offered a unique fingerprint of the dsRNA background released in the plot. Strains of *E. parasitica* carrying this specific dsRNA cytoplasmic background fit all the criteria necessary to be hypovirulent (7). Parts of this paper were reported elsewhere (8,9).

MATERIALS AND METHODS

Location. The experiment was conducted in an American chestnut grove at Crystal Lake near Frankfort, MI (15). The grove, consisting of approximately 3,000 trees, was infected with *E. parasitica* in the late 1970s, and was heavily infected with virulent strains of the fungus when the study was initiated.

Pentachloronitrobenzene (PCNB) resistance. A culture of *E. parasitica* capable of growing in the presence of PCNB was isolated and used in this study as follows (Table 1). Mycelial disks of PCNB-sensitive ($PCNB^S$) *E. parasitica* strain CL1 were placed on Endothia complete agar (ECA) modified by the omission of glucose (12) and containing either 100 μg of PCNB (Olin Terracoat L205) per milliliter (L-PCNB) or on ECA containing 1,000 μg of PCNB per milliliter (H-PCNB). After several days, a sector consisting of rapidly growing mycelium appeared. Mycelia from this sector were subcultured and tested for growth on ECA, L-PCNB, and H-PCNB media and for virulence (7). Reversion to PCNB sensitivity was tested by making 10-fold serial dilutions with PCNB-resistant ($PCNB^R$) conidia and plating 0.1 ml of each dilution on ECA and L-PCNB.

Pieces of American chestnut branches (6 cm long × 1 cm in diameter) were autoclaved and placed in 100 × 15-mm sterile plastic plates with approximately 20 ml of potato-dextrose agar (PDA; Difco, Detroit, MI). Each plate was inoculated with one of three strains of *E. parasitica* (Table 1). Control plates were uninoculated. Cultures were incubated at approximately 24 C under fluorescent light with a 16-hr photoperiod. Plates were used as inoculum

TABLE 1. Strains of *Endothia parasitica* used in the study of the dissemination of virulent and hypovirulent forms of the fungus in Michigan

Strain	Virulence[a]	PCNB[b]	Description
CL1	V	S	Isolated from a normal, virulent canker at Crystal Lake near Frankfort, MI, in 1980.
CL1 $PCNB^R$	V	R	Isolated from CL1 strain.
CL1(GH2) $PCNB^R$	H	R	CL1 $PCNB^R$ converted with the dsRNA from the GH2[c] hypovirulent strain.

[a] V = virulent, no dsRNA; H = hypovirulent, specific dsRNA segments present in the cytoplasm.

[b] R = resistant to 100 μg pentachloronitrobenzene (PCNB) per milliliter and S = sensitive to 100 μg PCNB per milliliter.

[c] A hypovirulent strain isolated from Grand Haven, MI, in 1980. This isolate contained a unique dsRNA banding pattern which was transferred to CL1 $PCNB^R$ by hyphal anastomosis (7).

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sources 2–3 wk after inoculation, when pycnidia had densely covered the wood and agar surfaces.

In spring 1982, 20 disease-free trees ranging from 12.1 to 29.7 cm in diameter were selected and divided into five blocks based upon trunk diameter 1 m above the soil level (Fig. 1). On 1 June 1982, one of the three inoculum sources or an uninoculated control plate was placed on the trunks at 2 m above the soil level. On the same date, 25 wounds were made in each of the twenty experimental trees. Five wounds were made in five rows 10, 35, 60, 85, and 110 cm below the inoculum source. Within each row, five types of sapwood wounds were made 3 cm apart in random order (Fig. 2). The five wound types were: cork borer hole, 5-mm diameter; nail hole, 3-mm diameter; scratch made by hammer claws, approximately 2 cm long; vertical scalpel slice, 1.5-cm long; and two scalpel slices (1.5 cm each) made at right angles with the apex pointing upward (inverted "V"). If a natural branch scar was in the vicinity, the natural branch scar was substituted for the latter type of wound. The inoculum sources were sprayed briefly with tap water to initiate conidial spread. Inoculum sources were replaced 25 August 1982 and 15 October 1982, and permanently removed on 7 December 1982.

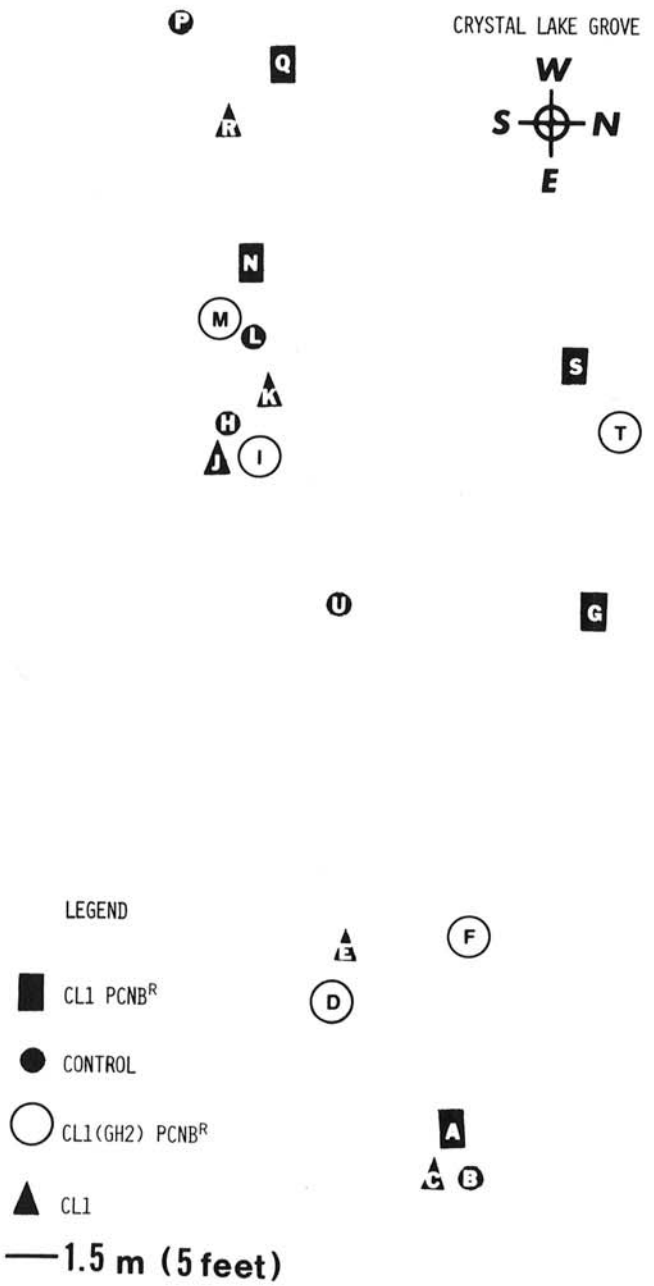

Fig. 1. Map showing the relative position of American chestnut trees and the various inoculum sources of *Endothia parasitica* placed on those trees in the study area at Crystal Lake, MI. Trees were designated by letters. The inoculum sources were: CL1, a wild-type virulent isolate; CL1 PCNB^R, CL1 resistant to pentachloronitrobenzene; and CL1 (GH2) PCNB^R, the CL1 PCNB^R isolate converted by the GH2 hypovirulent strain.

Detection of strains. Bark tissue samples approximately 2×2×1 mm were aseptically collected at 2, 4, 6, 11, 13, and 15 mo after the initial deployment of inoculum. At each of the six collection dates, one bark sample was taken from the center wound of the middle row 60 cm below the inoculum source and from the outside margin of any visible cankers that had developed on each tree. Bark samples were surface sterilized by immersion in a 15% solution of Clorox® (NaOCl, 5.25%) for 3–5 min and placed on PDA. If *E. parasitica* was isolated it was subcultured onto fresh PDA. Cultures were incubated as described above.

Isolates of *E. parasitica* recovered from bark samples were subcultured on ECA, L-PCNB, and H-PCNB. Cultures were incubated for 7 days as described above. Resistance to PCNB was determined by comparing the growth of the field isolates to that of standard strains CL1, CL1 PCNB^R, and CL1(GH2) PCNB^R on L-PCNB, H-PCNB, and ECA.

Each isolate was analyzed for dsRNA according the method of Day et al (3) with the modifications specified by Dodds (4) and Fulbright et al (7).

RESULTS

The sensitivity or resistance of bark isolates of *E. parasitica* to PCNB was easily detected when their growth on ECA after 7 days was compared to their growth on L-PCNB medium (Fig. 3).

Fig. 2. Photograph of a representative inoculum source of *Endothia parasitica* tied above established wound sites on an American chestnut tree trunk. Arrows indicate three of the five rows of wounds made below the inoculum source.

Colony diameters of isolates sensitive and resistant to PCNB were inhibited >85% and <70%, respectively, when compared to their diameters on ECA. The same approximate number of conidia of CL1 PCNB^R germinated and grew on both ECA and L-PCNB which indicated that reversion was negligible.

Fifteen, 13, 18, 33, 55, and 84 individual field isolates were collected from routinely-monitored center wounds and all cankers present at 2, 4, 6, 11, 13, and 15 mo from the start of experiment, respectively (Table 2). Only 16 isolates were recovered from established cankers during the first three sampling dates. During that period, however, 30 isolates were recovered from the routinely monitored center wounds. Numbers of cankers nearly doubled every 2 mo during the last 4 mo of sampling, resulting in a total of 72 established cankers associated with the 500 experimental wound sites. Two trees had a maximum of nine cankers and six trees had over five cankers. Three trees had no observable cankers and five trees had only one canker each. Isolates of *E. parasitica* from cankers or center wounds were present on all but two trees. After 15 mo, 12 isolates of *E. parasitica* were still being recovered from the repeatedly sampled center wound but only one canker became established at that location. *E. parasitica* was not isolated from the center wound on eight other trees during the last sampling.

Both the PCNB susceptibility data and the presence of specific dsRNA segments in the cytoplasmic backgrounds of *E. parasitica* isolated from established cankers during the 15-mo sampling period indicated that the inoculum sources placed in the trees could provide inoculum to the wounds made below them (Table 3). The data indicated that the majority of the cankers tested were associated with PCNB^S isolates. Since both CL1 inoculum and the wild-type inoculum already in the grove were of this phenotype (PCNB^S) it is impossible to distinguish if cankers were established by one or the other. However, the majority of isolates obtained from cankers on trees inoculated with PCNB^R inoculum sources were PCNB^R without dsRNA. A PCNB^R strain without dsRNA was only recovered from one other established canker on a tree with another inoculum source (Tree C; Table 3). Six isolates of PCNB^R with dsRNA were obtained (Tree G; Table 3). It is impossible to determine if all of these cankers were initiated by inoculum from strains of PCNB^R that became converted with dsRNA after initiating the canker or if isolates of CL1 (GH2) PCNB^R from nearby trees initiated the cankers. In August 1982 and again in December 1982, one of the cankers sampled was associated only with a strain of PCNB^R without dsRNA, but in May 1983 the isolate recovered from this canker was PCNB^R and contained dsRNA (*unpublished*). Strains of PCNB^R were found on two trees without inoculum sources of CL1-PCNB^R at other sampling dates, but these were not recovered at the final sampling date.

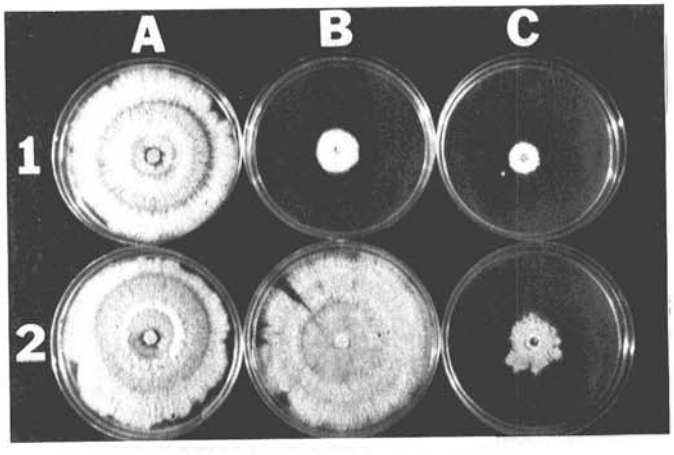

Fig. 3. Growth of isolates CL1 and CL1 PCNB^R of *Endothia parasitica* after 10 days on Endothia complete agar (ECA), ECA containing 100 μg of pentachloronitrobenzene (PCNB) per milliliter (L-PCNB), and ECA containing 1,000 μg of PCNB per milliliter (H-PCNB). Row 1, CL1; Row 2, CL1 PCNB^R. A, ECA; B, L-PCNB; and C, H-PCNB.

Strains of PCNB^R with dsRNA were found associated with a total of six cankers on trees with inoculum sources of CL1(GH2)PCNB^R. Two of the five trees with inoculum sources of CL1(GH2)PCNB^R had strains of PCNB^R with dsRNA associated with cankers while two trees only had one canker each from which strains of PCNB^S without dsRNA were recovered. The remaining tree (tree T), contained three undetected natural cankers at the beginning of the experiment. These natural cankers may have generated inoculum of PCNB^S that could have initiated cankers at the wound sites. This tree also contained the second highest number of cankers associated with strains of PCNB^S. Tree T was left in the study to determine the fate of the isolates of PCNB^S associated with the cankers. On two trees without a dsRNA inoculum source (trees E and G), dsRNA was found associated with cankers. On trees E and G, strains of PCNB^S with dsRNA were associated with one canker on each tree after 15 mo. Tree G is approximately 5 m from the closest source of CL1(GH2)PCNB^R.

The dsRNA detected in cultures of *E. parasitica* isolated during the first three sampling dates had dsRNA banding patterns in polyacrylamide gels that appeared to be identical to the unique dsRNA banding pattern of the hypovirulent inoculum source, CL1(GH2) PCNB^R (Table 4 and Fig. 4). Beginning in May 1983, different banding patterns began to appear, but specific similarities to the original banding pattern were maintained (Fig. 4). On the fourth, fifth, and sixth sampling dates, two, five, and nine isolates, respectively, were obtained that represented three distinct banding patterns (Table 4).

Differences between the number of isolates obtained from trees with respect to the four inoculum source types, and wound distance from the inoculum source were significant in May 1983, but not in July or September 1983 (Table 5). Differences between the number of isolates obtained from trees that possessed the phenotype of the

TABLE 2. The total number of cankers from which *Endothia parasitica* was isolated in relation to sampling dates. Numbers as listed are independent of the phenotype of the strain isolated from the canker

		Number of cankers from which *E. parasitica* was recovered from samples taken[a]					
		1982			1983		
Tree	Inoculum source[b]	Aug	Oct	Dec	May	July	Sept
A	CL1-PCNB^R	1	0†[c]	3	4	4†	7
B	Check	0	0	0†	0†	5†	7†
C	CL1	0†	0†	1	2†	2†	3†
D	CL1 (GH2) PCNB^R	0†	0†	2	4	5†	8†
E	CL1	0†	0†	0†	4†	7†	9†
F	CL1 (GH2) PCNB^R	0†	0†	0†	0	1	1†
G	CL1-PCNB^R	2†	1†	2†	3†	5†	9†
H	Check	0	0†	0	0	0	2†
I	CL1 (GH2) PCNB^R	0	0	0	0	0	0
J	CL1	0	0	0	0	0	1
K	CL1	0†	0	0	1	1†	1†
L	Check	0	0	0	0	0	0
M	CL1 (GH2) PCNB^R	0†	0†	0	0	0	1
N	CL1-PCNB^R	0†	0†	0	1†	1†	4
P	Check	0	0	0	0	0	0†
Q	CL1-PCNB^R	0†	0	0	1	1	1
R	CL1	0	0†	0†	1†	2†	4†
S	CL1-PCNB^R	0†	0†	2	2†	2†	4†
T[d]	CL1 (GH2) PCNB^R	0†	0†	2†	1†	6†	7
U	check	0†	0	0	0	1†	3†
Total		3(12)	1(12)	12(6)	24(8)	43(12)	72(12)

[a] The canker number may decline from a previous date if an isolate was not recovered due to contamination or lack of growth from a previously observed canker.

[b] Inoculum sources were placed on trees in June 1982 and removed in December 1982.

[c] † Indicates an isolate of *E. parasitica* was also recovered from the middle wound which was monitored whether or not a canker was observed.

[d] Three natural cankers with PCNB^S were discovered on this tree just after the beginning of the experiment.

inoculum source placed on the tree it was recovered from were not significant until September of 1983. Differences between the number of isolates obtained from trees with respect to the five wound types remained significant throughout 1983. Cankers were most frequently established at nail hole wounds. No significant interactions between all combinations of the three factors (inoculum source, wound distance, and wound type) were detected at any date.

By July 1983, 100% of the cankers had formed stroma. As a general observation, cankers associated with strains containing dsRNA had approximately 75% of the number of stroma observed in cankers not associated with dsRNA-containing strains. No perithecia were observed in cankers of this plot during this study, although they were present in other cankers in the grove.

DISCUSSION

We monitored the movement of hypovirulent *E. parasitica* by using a PCNB-resistant strain with a characteristic dsRNA banding pattern. Spread of virulent and hypovirulent strains within and between trees in the grove was detected. In addition, there appears to have been a transfer of dsRNA from the introduced strains of $PCNB^R$ containing dsRNA to strains of $PCNB^S$.

TABLE 3. Phenotypes, both nuclear and cytoplasmic, of isolates of *Endothia parasitica* recovered from established cankers on chestnut trees 15 mo after inoculation

Inoculum source[a]	Tree designation	Phenotype[b] of isolated strains			
		$PCNB^S$	$PCNB^S$/ dsRNA	$PCNB^R$	$PCNB^R$/ dsRNA
CL1-$PCNB^R$	A	0	0	7	0
	G	0	1	2	6
	N	0	0	4	0
	Q	0	0	1	0
	S	3	0	1	0
	Subtotals	3	1	15	6
CL1 (GH2) $PCNB^R$[c]	D	4	0	0	5
	F	0	0	0	4
	I	0	0	0	1
	M	1	0	0	0
	T[d]	7	0	0	0
	Subtotals	12	0	0	10
CL1	C	2	0	1	0
	E	8	1	0	0
	J	1	0	0	0
	K	1	0	0	0
	R	4	0	0	0
	Subtotals	16	1	1	0
Check	B	7	0	0	0
	H	2	0	0	0
	L	0	0	0	0
	P	0	0	0	0
	U	3	0	0	0
	Subtotals	12	0	0	0
Total		43	2	16	16

[a] Inoculum sources were placed on trees in June 1982 and removed in December 1983.
[b] $PCNB^S$, pentachloronitrobenzene-sensitive; $PCNB^R$, pentachloronitrobenzene-resistant.
[c] The unique CL1 (GH2) $PCNB^R$ double stranded ribonucleic acid banding pattern was present in the cytoplasmic background of the isolate.
[d] Three natural cankers with $PCNB^S$ inoculum were discovered on this tree just after the beginning of the experiment.

Transmission of dsRNA to new generations of *E. parasitica* has been shown to occur through the production of conidia containing dsRNA (16) and the transmission of dsRNA to other strains of *E. parasitica* probably occurs through hyphal anastomosis (3). Conidia have been shown to be disseminated by rain, insects, birds, and small mammals (1,2,10,11,13,14,17). There was no reason to suspect that the dissemination of dsRNA-containing strains should not occur in the same manner. To substantiate this possibility, Fulbright isolated dsRNA-containing conidia of *E. parasitica* from rain water below a natural canker in a recovering American chestnut grove in MI (D. W. Fulbright, *unpublished*). Therefore, in

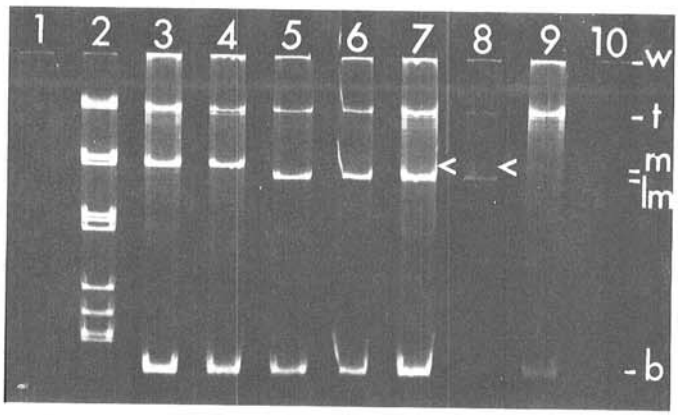

Fig. 4. Patterns of dsRNA segments from six field isolates of *Endothia parasitica* (all collected in September 1983), the three inoculum sources of *E. parasitica* used in this study, and a reovirus (REO3) mixed with a mycovirus from *Helminthosporium maydis* (Hm9), separated by electrophoresis on a 5% polyacrylamide gel (12 hr, 40 V) and stained with ethidium bromide (0.05 mg/ml H_2O). Lane 1, CL1; lane 2, REO3; lanes 3 and 4, type A, (Table 4) pattern equivalent to CL1(GH2) $PCNB^R$; lanes 5 and 6, type C pattern (Table 4) with only the new lower middle band; lanes 7 and 8, type B pattern (Table 4) with both middle band (very faint) and new lower middle band; lane 9, type D pattern (Table 4) with no middle band detectable; and lane 10, CL1 $PCNB^R$, no dsRNA detectable. W = bottom of well, t = top band, m = middle band, lm = lower middle band, and b = bottom band.

TABLE 4. Chronological history of dsRNA banding patterns found in isolates of *Endothia parasitica* containing changes in the GH2 dsRNA banding pattern

Isolated culture[a]	Type of dsRNA banding pattern observed at sampling date[b]:					
	1982			1983		
	Aug	Oct	Dec	May	July	Sept
D(R2-3)	–[c]	–	A	C	C	C
F	A	ND[d]	A	–	–	D
F(R3-5)	–	–	–	–	C	B
G(R1-3)	–	–	–	–	B	C
G(R3-5)	–	–	–	–	D	D
G(R4-2)	–	–	–	–	–	B
G(R4-4)	–	–	–	B	B	C
G(R5-1)	–	–	–	–	–	C
G(R5-4)	V[e]	–	V	A	A	B

[a] Information enclosed in parenthesis after the tree letter indicates location of canker on the tree; for example, D(R2-3) indicates the canker was present on tree D, row 2, wound site 3. If parentheses do not follow the tree letter then the isolated culture originated from bark around the routinely-monitored center wound of the third row.
[b] Banding patterns of dsRNA observed after polyacrylamide gel electrophoresis. A, CL1 (GH2) $PCNB^R$ banding pattern (Fig. 4, lane 3); B, contains a new dsRNA segment lower than the middle band (Fig. 4, lanes 7 and 8, faint); C, contains the new lower middle segment but lacks the original segment (Fig. 4, lane 5); and D, lacks both middle sigments (Fig. 4, lane 9).
[c] *Endothia parasitica* not isolated.
[d] ND, banding pattern of the isolate not determined.
[e] V, no dsRNA detected in recovered isolates.

this study we relied on inoculum spreading naturally from the inoculum source tied to each of the inoculated trees.

Hypovirulent strain CL1(GH2) PCNB^R was chosen for this study because it sporulates well and is more virulent than other hypovirulent strains (7). Moreover, the dsRNA is found in approximately 50% of the conidial lines tested (*unpublished*). Another desirable characteristic of this strain is that its dsRNA banding pattern is unique compared to the dsRNA banding patterns of other hypovirulent strains isolated in MI, thus making the dsRNA banding pattern easily recognizable on polyacrylamide gels (4,7).

When an isolate of *E. parasitica* was recovered that had the same phenotype as that of the inoculum source placed on that tree, it was assumed that the inoculum had originated from the artificial canker placed on the tree. It is possible, however, that isolates of PCNB^S without dsRNA could have originated from either CL1 inoculum or from the natural wild-type inoculum already present in the grove.

The inoculum sources effectively delivered inoculum to the wound sites beneath them. On check trees without inoculum sources, cankers were not observed until the fifth sampling date. However, on trees with inoculum sources, cankers were generally observed earlier and, in three cases, on the first sampling date (Table 2). Isolates collected from the center wound also indicated that inoculum was more readily prevalent on trees with inoculum sources even when cankers were not observed.

The recovery of isolates not sharing the same phenotype as the inoculum source placed on the tree is most important since this demonstrates the movement of *E. parasitica* and dsRNA between trees (Table 3). Because strains of PCNB^S with dsRNA were not introduced into the grove and because there is essentially no reversion to wild type by conidia from strains of PCNB^R, it appears that isolates of PCNB^S without dsRNA were converted by the transfer of dsRNA from dsRNA-containing strains on the trees.

All of the dsRNA banding patterns observed had the original GH2 top (T) and bottom (B) dsRNA segments intact, thus providing evidence that the dsRNA originated from CL1(GH2) PCNB^R (Fig. 4). However, the middle segment (M) appeared to be unstable. Banding pattern B (Table 4) had an additional dsRNA segment (LM), which was slightly smaller than M; this segment may have been derived from a 200-base-pair deletion of M (*unpublished*). For a period of time, both bands could have coexisted, then M could have been completely lost, leaving pattern C. Later, pattern D could have appeared when LM was lost. Chronological data tend to support this possibility (Table 4).

Most natural cankers found on American chestnut trees at the Crystal Lake site were located primarily at branch sites or scars (S. W. Garrod, *unpublished*). The wound type versus infection results of this study demonstrated that nail and cork borer holes, were more likely to have become infected. At the completion of the experiment, the chances of wounds at all distances becoming infected were statistically identical; earlier in the study, significant differences were found and a trend suggested that wounds closer to the inoculum source had a greater chance of being infected (Table 5). In general, these data demonstrate that inoculum can somehow be moved at least 110 cm down a tree trunk, encounter a wound, and initiate a canker (if the inoculum for these infections originated from the inoculum source on the same tree) and that the susceptibility of the host plant is homogeneous within the range of distance of the wounds on the trunk.

The control trees, in which no inoculum was placed, developed 12 cankers during the 15-mo study. This could be considered an approximate background infection level and if subtracted from the number of dsRNA-free isolates of PCNB^S associated with cankers on trees in which the other inoculum sources were placed would mean that most of the cankers associated with isolates in this category were from the natural surroundings. If this was the case, then CL1(GH2)PCNB^R and CL1-PCNB^R probably spread as well as the wild-type virulent strain, CL1. However, it would also point out the rapid rate of spread expected from natural inoculum. The continuous increase of cankers associated with PCNB^S strains masked differences that had previously existed (Table 5). If inoculum sources had been continually present there may have been a larger number of cankers on trees with inoculum sources than on controls.

To determine the fate of existing and new cankers, data will continue to be collected in this plot as long as the trees live.

TABLE 5. Number of cankers caused by inoculum of various strains of *Endothia parasitica* placed on American chestnut trees at various distances from standardized sapwood wounds

	Strain of *E. parasitica* placed on the tree				
Sampled (1983)	CL1	CL1 PCNB^R	CL1 (GH2) PCNB^R	None	χ^2
May	8	11	5	0	11.000*[a]
July	12	13	12	6	2.860
Sept	18	25	17	12	4.778

	Inoculant phenotype recovered from same tree			
	CL1	CL1 PCNB^R	CL1 (GH2) PCNB^R	χ^2
May	8	6	2	1.333
July	10	7	2	5.158
Sept	16	15	5	6.167*

	Sapwood wound types on inoculated trees					
	Cork borer	Nail	Scratch	Slice	Inverted "V" or branch scar	χ^2
May	5	18	0	0	1	48.916*
July	8	25	5	1	4	42.000*
Sept	17	32	10	6	7	32.027*

	Inoculum-to-wound distance down the trunk					
	10 cm	35 cm	60 cm	85 cm	110 cm	χ^2
May	9	8	3	2	2	9.750*
July	12	12	10	6	3	7.349
Sept	20	19	12	14	7	7.861

[a] Asterisk indicates differences between strains, phenotypes, wound types, or wound distances are significant at $P = 0.05$ by χ^2 test.

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