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DEPLOYMENT, DETECTION AND ANALYSIS OF HYPOVIRULENT STRAINS OF *ENDOTHIA PARASITICA* IN MICHIGAN

By

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ABSTRACT

DEPLOYMENT, DETECTION AND ANALYSIS
OF HYPOVIRULENT STRAINS OF
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The infection and spread of *Endothia parasitica* was examined in an American chestnut (*Castanea dentata*) grove using genetically marked strains of the fungus and a unique dsRNA banding pattern. Spread of virulent and hypovirulent strains of the fungus was detected within and among trees. There also appears to have been conversion *in situ* of virulent to hypovirulent strains. Infection studies suggest that nail and cork borer wounds were more likely to become infected than were others, however, cankers at branch scar wounds were largest in area. Wounds located 10 to 110 cm from inoculum sources were equally infected, but cankers nearer to the base of the tree were larger than those higher. Sexual mating types (A, and a) were determined for a number of Michigan isolates. dsRNA was not detected in ascospores isolated from perithecia formed by hypovirulent cultures.

To the memory of my grandmother,

Olive Colvin Sheltraw

ACKNOWLEDGEMENTS

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GENERAL INTRODUCTION AND LITERATURE REVIEW

The American chestnut (Castanea dentata [Marsh.] Borkh.), a member of the beech family (Fagaceae), was once a dominant or codominant species throughout the deciduous forests of eastern North America (7, 19, 34, 86). Its natural range extended from southern Maine south to southwest Georgia, west to southeastern Michigan, Ohio, Indiana, Kentucky, Tenessee and Mississippi (19, 34, 35, 65).

The American chestnut was a large tree with a massive trunk and a broad, rounded, dense crown. Its height and diameter ranged from 18-30 m and 0.6-1.2 m, respectively (65). Many useful products were obtained from this versatile tree. Wood was attractive, strong and unusually resistant to decay. Tannins extracted from the bark and wood were the basis of a large industry. Food for wildlife and man was furnished through its abundant production of nuts.

The average American chestnut tree of modern times is very different than that of the past. C. dentata now exists within its natural range primarily as sprouts growing from the root systems of trees that have been attacked by a fungal disease known as chestnut blight

(chestnut bark disease) (2, 35, 41, 52, 85, 86). The fungus was presumably introduced into North America around the turn of the twentieth century on Oriental chestnut stock imported from Asia. The pathogen, first found in North America in 1904 on trees in the New York Zoological Park in New York City (68), spread quickly throughout the chestnut forests of North America, reducing C. dentata to a minor understory shrub within fifty years (52).

The fungus, Endothia parasitica (Murr.) And. and And., an ascomycete (Diaporthales, Diaporthaceae), is the pathogen responsible for the chestnut blight disease. It also attacks oaks, maples, hickory and occasionally other trees, but not nearly as severely as it attacks the American chestnut (19, 69, 77). E. parasitica achieves infection in the chestnut by penetrating the bark through wounds (74). Aggregated in mycelial fans (buff-yellow in color), the fungus advances rapidly through the host, killing bark tissues, the vascular cambium and outer sapwood (7, 19, 34, 74) (Changes produced in the host tissues following fungal invasion have been described in detail by Keefer [60]). Elliptical cankers, sunken below or swollen above the surrounding bark become apparent at infection sites. The cankers are orange-brown in color due to fungal stromata which erupt through the surface of the infected bark. Mycelia within the cankers spread until the branch or stem is completely girdled, causing wilting and death of the distal portion of the tree beyond the infection (2, 14, 19, 35). Suckers (water sprouts) usually

arise below the cankered areas, stimulated by the interference of nutrient transfer, but these suckers eventually become blighted by new infections (14, 34, 62). Drooping clusters of dead leaves (termed "flags") on dead branches and/or undersized burrs may be additional symptoms associated with the disease (14).

Perithecia and pycnidia of E. parasitica are borne in the orange reddish-brown stromata which are scattered thickly over the canker. The stromata average 1.8 mm diameter, and are about 1.3 mm high. When wet, uninucleate, oblong or cylindrical, hyaline thin-walled conidia, 1.3 x 3.6 µm in size, exude in slender curving yellow cirrhi (spore horns) from irregularly shaped pycnidia embedded in the stromatal tissues (13). The conidia, also refered to as pycndidiospores, asexual spores or summer spores are dispersed during the spring, summer and autumn months by rain, wind, insects, birds, and small mammals (19, 40, 49, 77, 82, 87). Conidia germinate by two germ tubes within 12 to 36 hours in chestnut wounds, on PDA and almost any other nutrient media (13) (Bazziger has noted that biotin, in combination with an inorganic nitrogen source, and thiamine are essential for conidial germination [15]). Light is necessary for conidial production (13). Longevity of the spores has been determined to be at least one year (13). Neither freezing nor dessication was found to affect spore viability (49).

Perithecia of E. parasitica form in the autumn months, deeply embedded at the base of the stromata in

which the pycnidia are contained. Perithecia are globose, 350-400 µm in diameter, with long black necks that terminate at the surface of the stroma in an ostiole. When wet, some of the club-shaped asci within the perithecia expand, migrate towards the perithecial ostiole and forcibly eject oval, hyaline, two-celled thick-walled ascospores year-round (13, 50, 51, 72, 73, 74). The ascospores, which average approximately 8.6 x 4.5 µm are dispersed primarily by wind (12, 13, 74). Each of the two cells of the ascospore carries two to four nuclei (13). Ascospores will germinate within six to twelve hours (at approximately 24 C) by two germ tubes per cell in chestnut wounds, in water, or on any ordinary media (13). Longevity of the ascospores was found to be the same as for conidia (49).

Perithecia may also be produced in controlled laboratory crosses on autoclaved chestnut stems (3). Through some of these crosses it was determined that E. parasitica is homothallic, but will outcross preferentially (3, 4, 71). And, that more than one male nucleus (conidium) may fertilize a single protoperithecium (8).

Conventional methods such as sanitation, chemical application and breeding for resistance (through crosses involving the moderately and highly resistant Chinese and Japanese chestnut species, Castanea mollissima Bl. and Castanea crenata Sieb. and Zucc. have so far been ineffective in controlling the chestnut blight disease (35, 39, 59). The goal of resistance breeding programs has been

to produce a tree with the form of the American chestnut and the resistance of the Oriental species (the Oriental species have forms resembling apple trees). Progress has been made, but the desired endproduct has not yet been achieved (29, 53, 54, 64). Evidence of the existence of naturally occurring blight resistant American chestnuts has not been observed until recently (46, 47).

In Europe, Endothia parasitica was discovered in Italy in 1938 on Castanea sativa , Mill., the European chestnut (21). This tree, like the American chestnut is highly susceptible to chestnut blight (2). It is most likely that imported Oriental chestnut stocks were the source of these infections as was the case in the United States (35). Within 25 years of its discovery, the pathogen had spread to all major chestnut growing areas in Italy (66). Attempts to control the disease failed, as they had in the United States (35).

In 1950, Biraghi (22, 23) observed abnormal cankers on chestnut sprouts. The cankers were abnormal in that the fungus was restricted to the outer layer of the bark. As time passed, the number of abnormal cankers increased as the incidence of normal cankers decreased. Biraghi felt that the cankers were abnormal because the host had aquired a resistance to the disease (24).

Chestnut blight was discovered in France in 1965 (35). Grente acquired bark samples from abnormal cankers from Italy to study the phenomenon decribed by Biraghi (42). Grente and Sauret (43) found that many of the strains

isolated from the Italian abnormal cankers were different that those isolated from normal cankers. Cultures obtained from abnormal cankers were white (instead of the normal orange), sporulated less, and were less virulent than cultures obtained from normal cankers. In addition, these abnormal cultures slowed or prevented canker development when inoculated into chestnut bark together with normal cultures. Over a period of time, trees inoculated with the less virulent cultures recovered. Grente and Sauret (43) coined the term "exclusive hypovirulence" for the observed phenomenon because the abnormal cultures excluded the normal cultures by converting them to abnormal cultures. The mechanism by which these hypovirulent strains prevented the attack of more virulent strains was found to involve the transfer of cytoplasmic agents through hyphal anastomosis (20, 44, 86). Because of the existence of naturally occurring hypovirulent strains, chestnut blight is no longer a problem in Italy (66, 84) and is being controlled in France by a biological control program which involves the spread of artificially introduced hypovirulent strains (45).

The first native American hypovirulent strains were isolated from an abnormal canker on an American chestnut tree growing in Michigan (36). Additional native hypovirulent strains have been isolated from abnormal cankers on chestnuts located in other areas in Michigan as well as in Tennesse, Pennsylvania, Maryland, New York and Virginia (35, 58). These native American hypovirulent

strains behave as European strains, but differ in that the American strains are orange and the European are white when grown in culture (36, 38).

Hypovirulent strains (but not virulent strains) of Endothia parasitica , both European and American, were found to contain double-stranded ribonucleic acid (dsRNA) (30, 67), the genetic material of most fungal viruses (25). European and North American hypovirulent strains were found to contain one of at least three distinct types of complex dsRNA banding patterns (30, 31). Fulbright et al. (38), have reported several additional dsRNA banding patterns, different than those reported by Dodds, associated with hypovirulent strains isolated from several locations in Michigan.

Conversion of virulent strains to hypovirulent is accompanied by the transmission of dsRNA after hyphal anastomosis (5, 9, 30). Reversion back to full virulence by single conidial spore selection may be associated with loss of dsRNA (30). These findings suggested that dsRNA may be responsible for hypovirulence in E. parasitica (30, 31). However, evidence that dsRNA is the cause of hypovirulence is correlative because cell-free transmission of dsRNA into virulent strains has not yet been accomplished (85).

Dodds (32) isolated and purified an extract of pleomorphic, club-shaped particles from a European hypovirulent isolate that contained dsRNA. The appearance of the particles resembled virus-like particles (VLPs)

isolated from diseased mushrooms (63). Dodds suggested that these particles were either a new fungal VLP, or a site of accumulation of the dsRNA. Similar VLPs that also contained dsRNA were extracted from another European hypovirulent isolate (28). Newhouse, et al. (70), using transmission electron microscopy, has observed spherical, membrane-bounded VLPs in thin sections of hyphal tips of a European hypovirulent strain, but not in virulent isolates. He suggested that they may have been responsible for hypovirulence in the hypovirulent isolate.

Hypovirulent strains obtained from Grente and hypovirulent strains derived from American virulent strains (through hyphal anastomosis) were used to attempt to control virulent cankers on American chestnut trees in the United States (10, 55, 86). Cankers were more likely to be controlled by hypovirulent strains which were related. For example, French hypovirulent strains achieved control of cankers initiated by French virulent strains and American virulent strains converted to hypovirulent by European dsRNA controlled cankers initiated by American virulent strains. Cankers initiated by American virulent strains were not controlled by French virulent strains.

Vegetative incompatibility in E. parasitica has been described by Anagnostakis (1). Vegetatively incompatible strains might explain the failure of certain cankers to be controlled due to the lack of transfer of cytoplasmic determinants by the failure of hyphal anastomosis to occur between incompatible strains (1, 11). Anagnostakis has

determined that vegetative incompatibility is heterogenic, and controlled by at least 7 loci (with two alleles at each locus) (4). However, evidence has been presented that indicates vegetative incompatibility is not necessarily a barrier for hyphal anastomosis. Hypovirulent and virulent strains which belong to different vegetative compatibility groups can still fuse and transfer dsRNA (1). In addition, the problem of vegetative incompatibility in treating virulent cankers may be overcome by applying mixtures of hypovirulent strains that belong to many different vegetative compatibility groups (57).

Control of individual cankers on American chestnut trees has been achieved in many cases (11, 17, 55, 56, 57, 62, 86), however, due to secondary blight infections that developed later, trees continued to die. In Michigan however, American chestnut trees in some groves are surviving (26, 37, 38). This appears to be due to the presence of naturally occurring hypovirulent strains (37, 38).

Effective natural spread of hypovirulent strains of Endothia parasitica is essential if the biological control of chestnut blight is to succeed. Elliston has suggested that persistent sources of hypovirulent strains would increase the opportunity for spread (35). The strategy in the past has been to eliminate virulent cankers through treatment with hypovirulent strains. This process not only eliminates the virulent canker, but also the hypovirulent strains (because hypovirulent cankers are often healed over

time), thus removing the source of hypovirulent inoculum which is necessary for spread (35). Willey (88) has reported the spread of hypovirulence among cankers on trees that had been previously inoculated with hypovirulent strains. This procedure was successful in establishing hypovirulent strains on the same tree, but presented no evidence of spread to cankers on untreated trees.

The objectives of this research were: 1) to examine the spread of *E. parasitica* within a blighted American chestnut grove in Michigan using a genetically marked strain; 2) to study the infection of the American chestnut by *E. parasitica* based upon three physical factors (inoculum strain type, wound type and wound distance from inoculum source); 3) to determine the mating types of a number of Michigan *E. parasitica* isolates; and 4) to search for dsRNA in ascospores isolated from perithecia formed by hypovirulent cultures.

PART I

A. DEPLOYMENT AND DETECTION OF *ENDOTHIA PARASITICA* STRAINS WITHIN A BLIGHTED AMERICAN CHESTNUT GROVE IN NORTHERN MICHIGAN.

B. INFECTION OF THE AMERICAN CHESTNUT BASED UPON THREE FACTORS: (1) INOCULUM STRAIN TYPE, (2) WOUND TYPE AND (3) WOUND DISTANCE FROM INOCULUM SOURCE.

INTRODUCTION

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.). Today this blight continues killing young stump sprouts throughout New England and the Appalachian forest (34). In Europe and in certain groves in Michigan, chestnut trees survive and produce nuts in spite of the presence of chestnut blight (37, 38). It is hypothesized that hypovirulent strains of E. parasitica are responsible for the survival of these trees. Hypovirulent strains of the fungus are less virulent than normal strains, contain double-stranded ribonucleic acid (dsRNA) and can arrest individual cankers on trees which were caused by virulent strains.

Control of this disease may be possible through the use of hypovirulence. However, natural dissemination of the hypovirulent strains within groves is imperative if control is to be effectively achieved.

Willey (88) has reported the spread of hypovirulence among cankers on trees that had been previously inoculated with hypovirulent strains. This procedure was successful in establishing hypovirulent strains on the same tree, but

presented no evidence of spread to cankers on untreated trees.

The objectives of this research were to (a) examine the spread of *E. parasitica*, both hypovirulent and virulent, within a blighted American chestnut grove using genetically marked strains of the fungus and a known dsRNA banding pattern and (b) to study the infection of the American chestnut by *E. parasitica* based upon three physical factors: 1) inoculum strain type, 2) wound type and 3) wound distance from inoculum source.

MATERIALS AND METHODS

An American chestnut grove consisting of approximately 3000 trees was used to carry out the experiment. The stand, located at Crystal Lake near Frankfort, Michigan (83), is presently heavily infected with virulent strains of *E. parasitica* .

Fungal strains

Table 1 lists *Endothia parasitica* strains used in the study.

Deployment of the strains

Six by one cm sections of autoclaved chestnut wood (with bark) were placed in 100 x 15 mm sterile plastic plates with approximately 20 ml potato dextrose agar (PDA; Difco; Detroit, MI). Each plate was inoculated with one of three strains of *E. parasitica* :

(1) CL1 (virulent)

(2) CL1 PCNB-R (virulent and pentachloronitrobenzene [PCNB; Terra-Coat LT-2] resistant)

Table 1. *Endothia parasitica* strains used.

Strain	Virulence[1]	PCNB[2]	Description
CL1	V	S	Isolated from a normal, virulent canker at Crystal Lake, MI in 1980.
CL1 PCNB-R	V	R	Isolated from CL1 strain growing on PDA with 100 µg PCNB/ml.
CL1(GH2) PCNB-R	H	R	CL1 PCNB-R converted with the dsRNA from the GH2[3] hypovirulent strain.

[1] V = Virulent; H = Hypovirulent

[2] R = Resistant to pentachloronitrobenzene;
S = Sensitive to pentachloronitrobenzene

[3] A hypovirulent strain isolated from Grand Haven, MI in 1980. This isolate contains a unique dsRNA banding pattern which was transfered to CL1 PCNB-R by hyphal anastomosis.

(3) CL1(GH2) PCNB-R (hypovirulent and PCNB resistant)

Control plates were uninoculated. Cultures were incubated at approximately 24 C under fluorscent lights with a 16-hr photoperiod.

Two to three weeks later, after pycnidia were densely covering the wood and agar surfaces, two small holes were drilled on both sides of the wood through the agar and plastic. The cover and sides of the plates were removed and cotton string was threaded through the holes so that the plates could be tied to the tree trunks (Fig. 1).

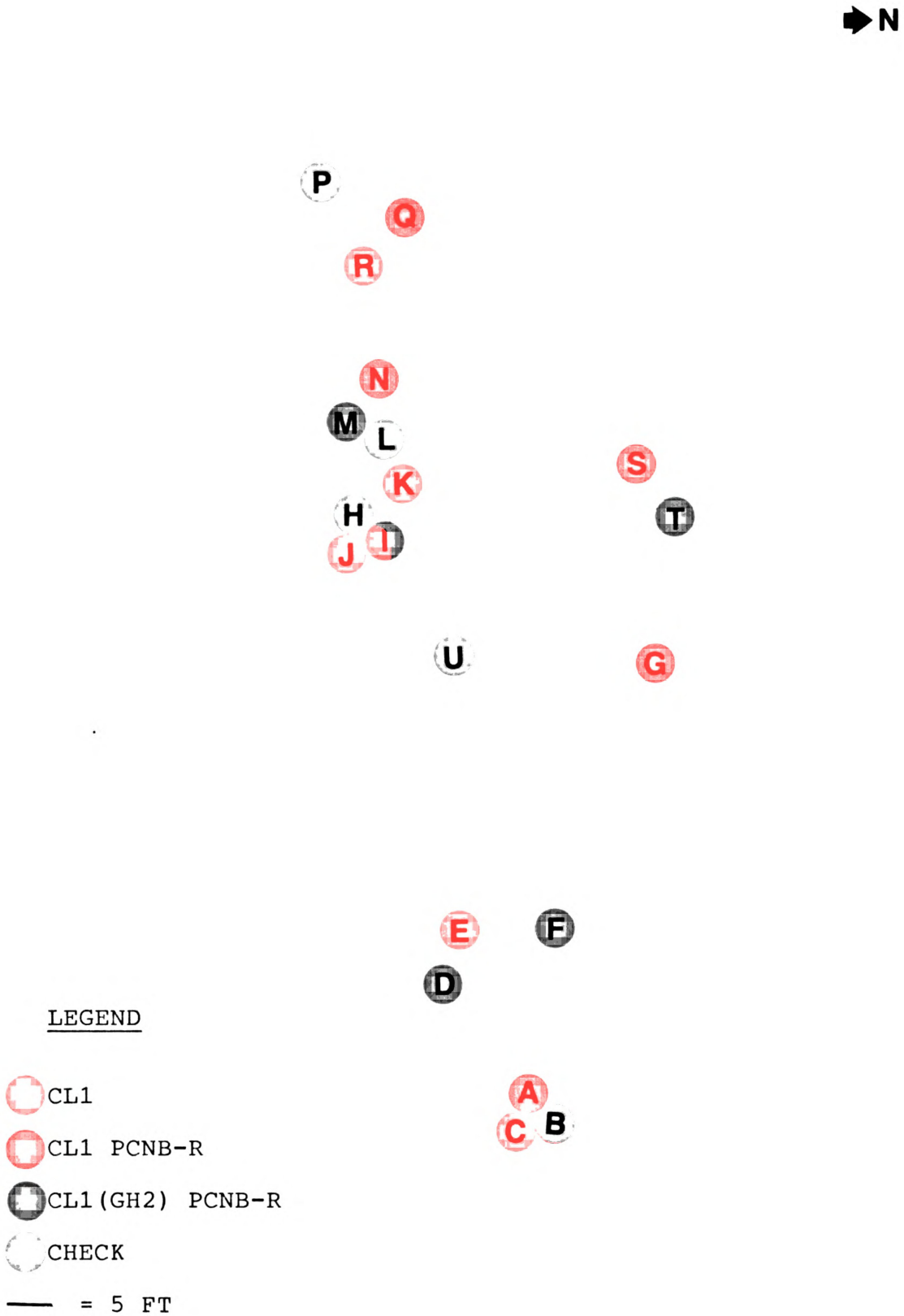
In June 1982, twenty trees (Fig. 2) were selected and divided into five blocks based upon trunk diameter at one meter above soil level (trees chosen ranged from 12.13 to 29.71 cm in diameter). Five wound types:

(1) cork borer hole (5 mm diameter)
(2) nail hole (3 mm diameter)
(3) scratch (approximately 2 cm [made with hammer tines])
(4) vertical scalpel slice (1.5 cm)
(5) natural or artificial branch scar (Artificial branch scars were made by two scalpel slices [1.5 cm each] at right angles to each other with the apex of the angle pointing towards the top of the tree.)

were made to the depth of the sapwood, 3 cm apart in a

Figure 1. Photograph of a representative E. parasitica inoculum source tied over established wound sites on an American chestnut tree trunk.

Figure 2. Map showing the relative position of trees used in the study.


N
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T
U
G
E
F
D
A
C
B
LEGEND
CL1
CL1 PCNB-R
CL1(GH2) PCNB-R
CHECK
= 5 FT

FIGURE 2

randomized order at five distances:

(1) 10 cm
(2) 35 cm
(3) 60 cm
(4) 85 cm
(5) 110 cm

from two meters above soil level on each tree trunk. One of the four inoculum sources (CL1, CL1 PCNB-R, CL1(GH2) PCNB-R, and control) was placed on the trunks over the wound series at two meters above soil level (Fig. 1). The inoculum sources were sprayed briefly with tap water to initiate conidial spread. Inoculum sources were replaced by fresh ones in August 1982 and October 1982, and removed in December 1982.

Pieces of bark tissue approximately 2 mm^2 were aseptically collected 2, 4, 6, 11, 13 and 15 months later from the center wound of the middle row and from any visible cankers that had developed on each tree. At the time of the final three samplings, canker areas were determined (from length and width measurements using the formula for an ellipse) and the presence of pycnidia and perithecia was noted (as either "+" fruiting bodies present or "-" fruiting bodies absent). Bark samples were immersed in 15% commercial Chlorox (NaClO, 5.25%) solution 3-5 min. and placed on PDA. If E. parasitica was isolated it was subcultured onto fresh PDA. Cultures were incubated at

approximately 24 C under fluorescent lights with a 16-hr photoperiod.

Detection of strains

Each time E. parasitica was isolated it was subcultured on 2 plates of three different media 1) Endothia complete medium (ECM) (71), modified by the omission of glucose, 2) ECM with 100 µg PCNB/ml (Low-PCNB) and 3) ECM with 1000 µg PCNB/ml (High-PCNB). Cultures were incubated for seven days at approximately 24 C under fluorescent lights with a 16-hr photoperiod. Resistance to PCNB carried by isolates obtained from the trees was determined by comparing the percent area growth on L-PCNB and H-PCNB to ECM without PCNB, to percent area growth of standards (CL1, CL1 PCNB-R and CL1(GH2) PCNB-R on the same media.

Each isolate was analyzed for dsRNA after the method of Day et al. (30) with modifications by Dodds (31) and Fulbright (38).

RESULTS

Sixteen, fourteen, eighteen, thirty-five, fifty-eight and eighty-seven field isolates were collected from wounds and cankers after two, four, six, eleven, thirteen and fifteen months, respectively. PCNB resistance and dsRNA content was determined for all isolates that had not become contaminated in the laboratory (Tables 2 - 7).

PCNB-R virulent strains were recovered from trees in which CL1 PCNB-R was the source of inoculum. PCNB sensitive (PCNB-S) virulent strains were recovered from trees with CL1 sources of inoculum, and PCNB-R hypovirulent strains were isolated from trees with CL1(GH2) PCNB-R sources of inocula.

Isolates differing in PCNB sensitivity and virulence from the inoculum sources placed on trees were also detected. Fifty cultures, isolated from trees which had PCNB-R/virulent and PCNB-R/hypovirulent inoculum sources, were PCNB-S and virulent (Table 8). Four PCNB-R/virulent cultures were isolated from trees with PCNB-S/virulent and PCNB-R/hypovirulent inocula (Table 8). Seventeen PCNB-R/hypovirulent strains were found on trees with CL1 PCNB-R and CL1 as inoculum sources (Table 8). And five PCNB-S/hypovirulent strains were isolated from cankers on

Table 2. *Endothia parasitica* isolates obtained from designated wounds or cankers on study trees at Crystal Lake, MI in August 1982, and their sensitivity to PCNB and the presence of dsRNA.

Inoculum Source[1]	Culture Isolated	PCNB Resistance[2]	dsRNA[3]
CL1	T-C[4]	-	-
	T-E	-	-
	T-K	-	-
CL1 PCNB-R	T-A(R1-3)[5]	+	-
	T-G	+	-
	T-G(R2-1)	+	-
	T-G(R5-4)	+	-
	T-N	+	-
	T-Q	+	-
	T-S	+	-
CL1(GH2) PCNB-R	T-D	+	+
	T-F	-	+
	T-M	+	-
	T-T	-	-
	T-T(c#1)[6]	-	-
None	T-U	-	-

[1] *E. parasitica* strains CL1, CL1 PCNB-R or CL1(GH2) PCNB-R were grown on autoclaved chestnut segments in a petri dish with PDA. These were tied to designated trees within the study area.

[2] + = Resistance to PCNB; - = Sensitive to 100 µg PCNB/ml

[3] + = Presence of dsRNA; - = dsRNA not detected

[4] If parenthesis do not follow the tree letter then the isolated culture originated from the third wound site of the third row for the indicated tree (not a canker).

[5] Information enclosed in parenthesis after the tree letter indicates location of canker on the tree. The canker on this tree is located at the third wound site in the first row.

[6] T-T(c#1) was isolated from a natural canker (canker #1) that had begun development prior to the beginning of the experiment.

Table 3. *Endothia parasitica* isolates obtained from designated wounds or cankers on study trees at Crystal Lake, MI in October 1982, and their sensitivity to PCNB and the presence of dsRNA.

Inoculum Source[1]	Culture Isolated	PCNB Resistance[2]	dsRNA[3]
CL1	T-C[4]	-	c
	T-E	-	-
	T-R	-	-
CL1 PCNB-R	T-A	-	-
	T-G	+	c
	T-G(R2-1)[5]	+	-
	T-N	+	-
	T-S	+	-
CL1(GH2) PCNB-R	T-D	-	c
	T-F	-	c
	T-M	+	c
	T-T	-	c
	T-T(c#1)[6]	-	-
None	T-H	-	c

[1] *E. parasitica* strains CL1, CL1 PCNB-R or CL1(GH2) PCNB-R were grown on autoclaved chestnut segments in a petri dish with PDA. These were tied to designated trees within the study area.

[2] + = Resistance to PCNB; - = Sensitive to 100 µg PCNB/ml

[3] + = Presence of dsRNA; - = dsRNA not detected; c = Culture was contaminated before the presence of dsRNA could be tested.

[4] If parenthesis do not follow the tree letter then the isolated culture originated from the third wound site of the third row for the indicated tree (not a canker).

[5] Information enclosed in parenthesis after the tree letter indicates location of canker on the tree. The canker on this tree is located at the first wound site in the second row.

[6] T-T(c#1) was isolated from a natural canker (canker #1) that had begun development prior to the initiation of the experiment.

Table 4. *Endothia parasitica* isolates obtained from designated wounds or cankers on study trees at Crystal Lake, MI in December 1982, and their sensitivity to PCNB and the presence of dsRNA.

Inoculum Source[1]	Culture Isolated	PCNB Resistance[2]	dsRNA[3]
CL1	T-C(R2-1)[4]	-	-
	T-E[5]	-	-
	T-R	+	-
CL1 PCNB-R	T-A(R1-3)	+	-
	T-A(R2-2)	+	-
	T-A(R2-3)	+	-
	T-G	+	+
	T-G(R2-1)	+	-
	T-G(R5-4)	+	-
	T-S(R1-4)	-	c
	T-S(R3-5)	+	-
CL1(GH2) PCNB-R	T-D(R2-3)	+	+
	T-D(R2-5)	+	+
	T-F	+	+
	T-T	-	-
	T-T(R1-2)	-	c
	T-T(R5-3)	-	-
None	T-B	-	-

[1] *E. parasitica* strains CL1, CL1 PCNB-R or CL1(GH2) PCNB-R were grown on autoclaved chestnut segments in a petri dish with PDA. These were tied to designated trees within the study area.

[2] + = Resistance to PCNB; - = Sensitive to 100 µg PCNB/ml

[3] + = Presence of dsRNA; - = dsRNA not detected; c = Culture was contaminated before the presence of dsRNA could be tested.

[4] Information enclosed in parenthesis after the tree letter indicates location of canker on the tree. The canker on this tree is located at the first wound site in the second row.

[5] If parenthesis do not follow the tree letter then the isolated culture originated from the third wound site of the third row for the indicated tree (not a canker).

Table 5. *Endothia parasitica* isolates obtained from designated wounds or cankers on study trees at Crystal Lake, MI in May 1983, and their sensitivity to PCNB and the presence of dsRNA.

Inoculum Source[1]	Culture Isolated	PCNB Resistance[2]	dsRNA[3]
CL1	T-C[4]	-	-
	T-C(R2-1)[5]	-	-
	T-C(R4-1)	-	-
	T-E	-	-
	T-E(R1-2)	-	-
	T-E(R1-3)	-	-
	T-E(R2-3)	-	-
	T-E(R5-5)	-	-
	T-K(R2-1)	-	-
	T-R	-	-
	T-R(R3-4)	-	-
CL1 PCNB-R	T-A(R1-3)	+	-
	T-A(R2-2)	-	-
	T-A(R2-3)	+	-
	T-A(R3-4)	+	-
	T-G	+	+
	T-G(R2-2)	+	-
	T-G(R4-4)	+	+
	T-G(R5-4)	+	+
	T-N	+	-
	T-N(R1-2)	+	-
	T-Q(R1-2)	+	-
	T-S	+	-
	T-S(R1-4)	-	-
	T-S(R3-5)	-	-
CL1(GH2) PCNB-R	T-D	-	-
	T-D(R1-1)	-	-
	T-D(R1-5)	-	-
	T-D(R2-3)	+	+
	T-D(R2-5)	+	+
	T-T	-	-
	T-T(R1-2)[6]	-	-
	T-T(c#1)	-	-
	T-T(c#2)	-	-
None	T-B	-	-

[1] *E. parasitica* strains CL1, CL1 PCNB-R or CL1(GH2) PCNB-R were grown on autoclaved chestnut segments in a petri dish with PDA. These were tied to designated trees within the study area.

Table 5. (continued)

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[2]+ = Resistance to PCNB; - = Sensitive to 100 µg PCNB/ml

[3]+ = Presence of dsRNA; - = dsRNA not detected

[4]If parenthesis do not follow the tree letter then the isolated culture originated from the third wound site of the third row for the indicated tree (not a canker).

[5]Information enclosed in parenthesis after the tree letter indicates location of canker on the tree. The canker on this tree is located at the first wound site in the second row.

[6]T-T(c#1) and T-T(c#2) were isolated from natural cankers (numbered 1 and 2) that had begun development prior to the initiation of the experiment.

Table 6. *Endothia parasitica* isolates obtained from designated wounds or cankers on study trees at Crystal Lake, MI in July 1983, and their sensitivity to PCNB and the presence of dsRNA.

Inoculum Source[1]	Culture Isolated	PCNB Resistance[2]	dsRNA[3]
CL1	T-C[4]	-	-
	T-C(R2-1)[5]	-	-
	T-C(R4-1)	-	-
	T-E	-	-
	T-E(R1-1)	-	-
	T-E(R1-2)	-	-
	T-E(R2-3)	-	-
	T-E(R2-4)	-	-
	T-E(R3-2)	-	-
	T-E(R4-1)	+	+
	T-E(R5-5)	+	-
	T-K	-	-
	T-K(R2-1)	-	-
	T-R	-	-
	T-R(R3-4)	-	-
	T-R(R5-3)	-	-
CL1 PCNB-R	T-A	+	-
	T-A(R1-2)	+	-
	T-A(R1-3)	+	-
	T-A(R2-3)	+	-
	T-A(R3-4)	+	-
	T-G	+	+
	T-G(R1-3)	+	+
	T-G(R2-1)	+	-
	T-G(R3-5)	+	+
	T-G(R4-4)	+	+
	T-G(R5-4)	+	+
	T-N	+	-
	T-N(R3-5)	+	-
	T-Q(R1-2)	+	-
	T-S	+	-
	T-S(R1-4)	-	-
	T-S(R3-5)	-	-
CL1(GH2) PCNB-R	T-D	+	+
	T-D(R1-1)	-	-
	T-D(R1-5)	-	-
	T-D(R2-3)	+	+
	T-D(R2-5)	+	+
	T-D(R3-5)	-	-
	T-F(R3-5)	-	+
	T-T	-	-
	T-T(R1-1)	-	-
	T-T(R1-2)	-	-
	T-T(R1-5)	-	-
	T-T(R2-3)	-	-

Table 6. (continued)

Inoculum Source[1]	Culture Isolated	PCNB Resistance[2]	dsRNA[3]
CL1(GH2) PCNB-R	T-T(R3-5)	-	-
	T-T(R4-5)[6]	-	-
	T-T(c#1)	-	-
	T-T(c#2)	-	-
	T-T(c#3)	-	-
None	T-B	-	-
	T-B(R2-1)	-	-
	T-B(R2-4)	-	-
	T-B(R3-5)	-	-
	T-B(R4-1)	-	-
	T-B(R4-3)	-	-
	T-U	-	-
	T-U(R2-4)	-	-

[1] E. parasitica strains CL1, CL1 PCNB-R or CL1(GH2) PCNB-R were grown on autoclaved chestnut segments in a petri dish with PDA. These were tied to designated trees within the study area.

[2] + = Resistance to PCNB; - = Sensitive to 100 µg PCNB/ml

[3] + = Presence of dsRNA; - = dsRNA not detected

[4] If parenthesis do not follow the tree letter then the isolated culture originated from the third wound site of the third row for the indicated tree (not a canker).

[5] Information enclosed in parenthesis after the tree letter indicates location of canker on the tree. The canker on this tree is located at the first wound site in the second row.

[6] T-T(c#1), T-T(c#2) and T-T(c#3) were isolated from natural cankers (numbered 1, 2 and 3) that had begun development prior to the initiation of the experiment.

Table 7. *Endothia parasitica* isolates obtained from designated wounds or cankers on study trees at Crystal Lake, MI in September 1983, and their sensitivity to PCNB and the presence of dsRNA.

Inoculum Source[1]	Culture Isolated	PCNB Resistance[2]	dsRNA[3]
CL1	T-C[4]	-	-
	T-C(R1-5)[5]	-	-
	T-C(R2-1)	-	-
	T-C(R4-5)	+	-
	T-E	-	-
	T-E(R1-1)	-	-
	T-E(R1-2)	-	-
	T-E(R1-3)	-	-
	T-E(R2-3)	-	-
	T-E(R2-4)	-	-
	T-E(R3-2)	-	-
	T-E(R4-1)	-	+
	T-E(R4-5)	-	-
	T-E(R5-5)	-	-
	T-J(R1-5)	-	-
	T-K	-	-
	T-K(R1-2)	-	-
	T-R	-	-
	T-R(R2-4)	-	-
	T-R(R2-5)	-	-
	T-R(R4-1)	-	-
	T-R(R5-2)	-	-
CL1 PCNB-R	T-A(R1-2)	+	-
	T-A(R1-3)	+	-
	T-A(R2-1)	+	-
	T-A(R2-2)	+	-
	T-A(R2-3)	+	-
	T-A(R3-3)	+	-
	T-A(R3-4)	+	-
	T-G	+	+
	T-G(R1-1)	-	+
	T-G(R1-3)	+	+
	T-G(R2-1)	+	-
	T-G(R2-2)	+	-
	T-G(R3-5)	+	+
	T-G(R4-2)	+	+
	T-G(R4-4)	+	+
	T-G(R5-1)	+	+
	T-G(R5-4)	+	+
	T-N(R1-4)	+	-
	T-N(R3-5)	+	-
	T-N(R4-2)	+	-
	T-N(R5-1)	+	-
	T-Q(R1-2)	+	-
	T-S	-	-

Table 7. (continued)

Inoculum Source[1]	Culture Isolated	PCNB Resistance[2]	dsRNA[3]
CL1 PCNB-R	T-S(R1-4)	-	-
	T-S(R2-5)	-	-
	T-S(R3-5)	-	-
	T-S(R4-4)	+	-
CL1(GH2) PCNB-R	T-D	+	+
	T-D(R1-1)	-	-
	T-D(R1-5)	-	-
	T-D(R2-3)	+	+
	T-D(R2-5)	+	+
	T-D(R3-5)	-	-
	T-D(R4-2)	-	-
	T-D(R4-3)	+	+
	T-D(R5-2)	+	+
	T-F	+	+
	T-F(R3-5)	+	+
	T-M(R3-5)	-	-
	T-T(R1-1)	-	-
	T-T(R1-2)	-	-
	T-T(R1-5)	-	-
	T-T(R2-3)	-	-
	T-T(R3-5)	-	-
	T-T(R4-5)	-	-
	T-T(R5-3)	-	-
	T-T(c#1)[6]	-	-
	T-T(C#2)	-	-
	T-T(c#3)	-	-
None	T-B	-	-
	T-B(R1-4)	-	-
	T-B(R2-1)	-	-
	T-B(R2-4)	-	-
	T-B(R2-5)	-	-
	T-B(R3-5)	-	-
	T-B(R4-1)	-	-
	T-B(R4-3)	-	-
	T-H	-	-
	T-H(R2-5)	-	-
	T-H(R4-4)	-	-
	T-P	-	-
	T-U	-	-
	T-U(R1-4)	-	-
	T-U(R2-4)	-	-
	T-U(R3-4)	-	-

Table 7. (continued)

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[1] *E. parasitica* strains CL1, CL1 PCNB-R or CL1(GH2) PCNB-R were grown on autoclaved chestnut segments in a petri dish with PDA. These were tied to designated trees within the study area.

[2] + = Resistance to PCNB; - = Sensitive to 100 µg PCNB/ml

[3] + = Presence of dsRNA; - = dsRNA not detected

[4] If parenthesis do not follow the tree letter then the isolated culture originated from the third wound site of the third row for the indicated tree (not a canker).

[5] Information enclosed in parenthesis after the tree letter indicates location of canker on the tree. The canker on this tree is located at the fifth wound site in the first row.

[6] T-T(c#1), T-T(c#2) and T-T(c#3) were isolated from natural cankers (numbered 1, 2 and 3) that had begun development prior to the initiation of the experiment.

Table 8. Additional PCNB/virulence isolate types for *Endothia parasitica* cultures isolated from wounds and cankers.

Inoculum Source	PCNB/Virulence Status[1] of *E. parasitica* Isolated	Isolated Culture, (Sampling Date)
1) CL1 PCNB-R	S/V	T-A[2], (10/82)
		T-A(R2-2)[3], (5/83)
		T-S(R1-4), (5/83)
		T-S(R3-5), (5/83)
		T-S(R1-4), (7/83)
		T-S(R3-5), (7/83)
		T-S, (9/83)
		T-S(R1-4), (9/83)
		T-S(R2-5), (9/83)
		T-S(R3-5), (9/83)
2) CL1(GH2) PCNB-R	S/V	T-T, (8/82)
		T-T(c#1)[4], (8/82)
		T-T(c#1), (10/82)
		T-T, (12/82)
		T-T(R5-3), (12/82)
		T-D, (5/83)
		T-D(R1-1), (5/83)
		T-D(R1-5), (5/83)
		T-T, (5/83)
		T-T(R1-2), (5/83)
		T-T(c#1), (5/83)
		T-T(c#2), (5/83)
		T-D(R1-1), (7/83)
		T-D(R1-5), (7/83)
		T-D(R3-5), (7/83)
		T-T, (7/83)
		T-T(R1-1), (7/83)
		T-T(R1-2), (7/83)
		T-T(R1-5), (7/83)
		T-T(R2-3), (7/83)
		T-T(R3-5), (7/83)
		T-T(R4-5), (7/83)
		T-T(c#1), (7/83)
		T-T(c#2), (7/83)
		T-T(c#3), (7/83)
		T-D(R1-1), (9/83)
		T-D(R1-5), (9/83)
		T-D(R3-5), (9/83)
		T-D(R4-2), (9/83)
		T-M(R3-5), (9/83)
		T-T(R1-1), (9/83)
		T-T(R1-2), (9/83)

Table 8. (continued)

Inoculum Source	PCNB/Virulence Status[1] of *E. parasitica* Isolated	Isolated Culture, (Sampling Date)
2) CL1(GH2) PCNB-R	S/V	T-T(R1-5), (9/83) T-T(R2-3), (9/83) T-T(R3-5), (9/83) T-T(R4-5), (9/83) T-T(R5-3), (9/83) T-T(c#1), (9/83) T-T(c#2), (9/83) T-T(c#3), (9/83)
3) CL1	R/V	T-R, (12/82) T-E(R5-5), (7/83) T-C(R4-5), (9/83)
4) CL1(GH2) PCNB-R	R/V	T-M, (8/82)
5) CL1 PCNB-R	R/H	T-G, (12/82) T-G, (5/83) T-G(R4-4), (5/83) T-G(R5-4), (5/83) T-G, (7/83) T-G(R1-3), (7/83) T-G(R3-5), (7/83) T-G(R4-4), (7/83) T-G(R5-4), (7/83) T-G, (9/83) T-G(R1-3), (9/83) T-G(R3-5), (9/83) T-G(R4-2), (9/83) T-G(R4-4), (9/83) T-G(R5-1), (9/83) T-G(R5-4), (9/83)
6) CL1	R/H	T-E(R4-1), (7/83)
7) CL1	S/H	T-E(R4-1), (9/83)
8) CL1(GH2) PCNB-R	S/H	T-F, (8/82) T-F(R3-5), (7/83)
9) CL1 PCNB-R	S/H	T-G(R1-1), (9/83

Table 8. (continued)

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[1]R = Resistant to PCNB; S = Sensitive to PCNB;
V = Virulent; H = Hypovirulent

[2]If parenthesis do not follow the tree letter then the isolated culture originated from the third wound site of the third row for the indicated tree (not a canker).

[3]Information enclosed in parenthesis indicates location of canker on tree. The canker on this tree is located at the second wound site in the second row.

[4]T-T(c#1), T-T(c#2) and T-T(c#3) were isolated from natural cankers (numbered 1, 2 and 3) that had begun development prior to the initiation of the experiment.

three trees with CL1, CL1(GH2) PCNB-R and CL1 PCNB-R as inoculum sources (Table 8). Cultures which became contaminated before the presence of dsRNA could be tested were not included in Table 8.

The dsRNA detected in E. parasitica cultures isolated during the first three sampling dates had dsRNA banding patterns in polyacrylamide gels that were identical to the unique dsRNA banding pattern of the hypovirulent inoculum source [CL1(GH2) PCNB-R] (Figures 3 and 4). Beginning in May 1983, new, different banding patterns began to appear, however, they contained certain similarities to the original banding patterns (Figs. 3 and 4, Table 9). Two, five and nine isolates were obtained with new dsRNA banding patterns from the fourth, fifth and sixth sampling dates, respectively (Table 10). Three distinct banding patterns were found when observed after electrophoresis on 5% polyacrylamide gels (Figures 3 and 4).

Differences between the number of cankers initiated on trees with respect to inoculum source, and wound distance from the inoculum source were significant at P=0.05 by the X^2 (81) test in May, but not in July or September 1983 (Table 11). Differences between the number of cankers initiated on a tree whose isolated cultures were the same in regards to PCNB sensitivity and virulence as the inoculum source on the same tree were not significant until September of 1983 (Table 11). Differences between the number of cankers that developed at the various wound types

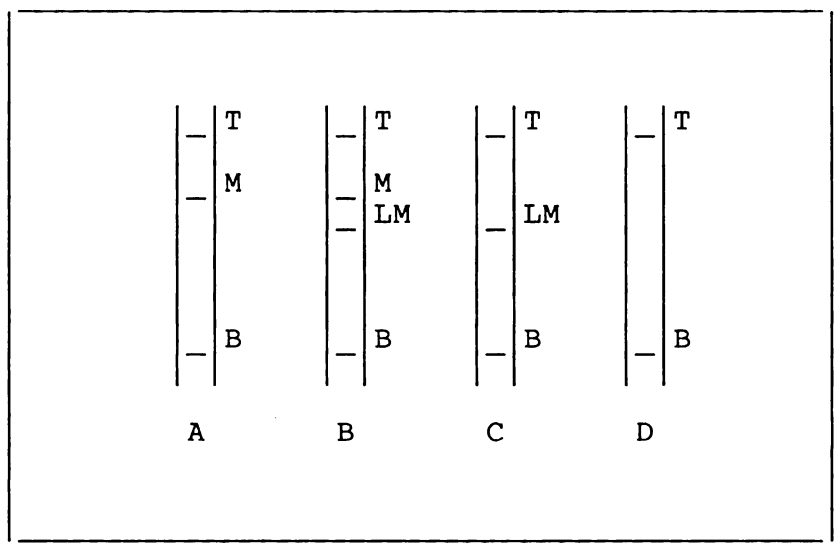


Figure 3. Diagram representing the dsRNA banding pattern observed in the CL1(GH2) PCNB-R strain in comparison to the banding patterns observed in the isolates with changes in the expected banding pattern. Horizontal lines labeled T, M, LM or B represent dsRNA segments separated by electrophoresis on 5% polyacrylamide gels (12 hrs, 40 V).

A. dsRNA banding pattern of CL1(GH2) PCNB-R, contains three main segments: top (T), middle (M) and bottom (B).

B. dsRNA banding pattern B, contains four main segments: top, middle, lower middle (LM), and bottom.

C. dsRNA banding pattern C, contains three main segments: top, lower middle and bottom.

D. dsRNA banding pattern D, contains two main segments: top and bottom.

Figure 4. Patterns of dsRNA segments from six E. parasitica field isolates (all collected 9-83), the three E. parasitica inoculum sources, and reovirus mixed with a VLP from Helminthosporium maydis (Hm9) separated by electrophoresis on a 5% polyacrylamide gel (12 hr, 40 V) and stained with ethydium bromide (0.05 mg/ml H_2O).

W = wells
T = top band
M = middle band
LM = lower middle band
B = bottom band

Lane	Sample
1	CL1
2	reovirus/Hm9
3	CL1(GH2) PCNB-R
4	T-E(R4-1)[1]
5	T-D(R2-5)
6	T-G(R1-1)
7	T-G(R4-2)
8	T-F(R3-5)
9	T-F[2]
10	CL1 PCNB-R

[1]Information enclosed in parenthesis after the tree letter indicates location of canker on the tree. The canker on this tree is located at the first wound site in the fourth row.

[2]If parenthesis do not follow the tree letter then the isolated culture originated from the third wound site of the third row for the indicated tree (not a canker).

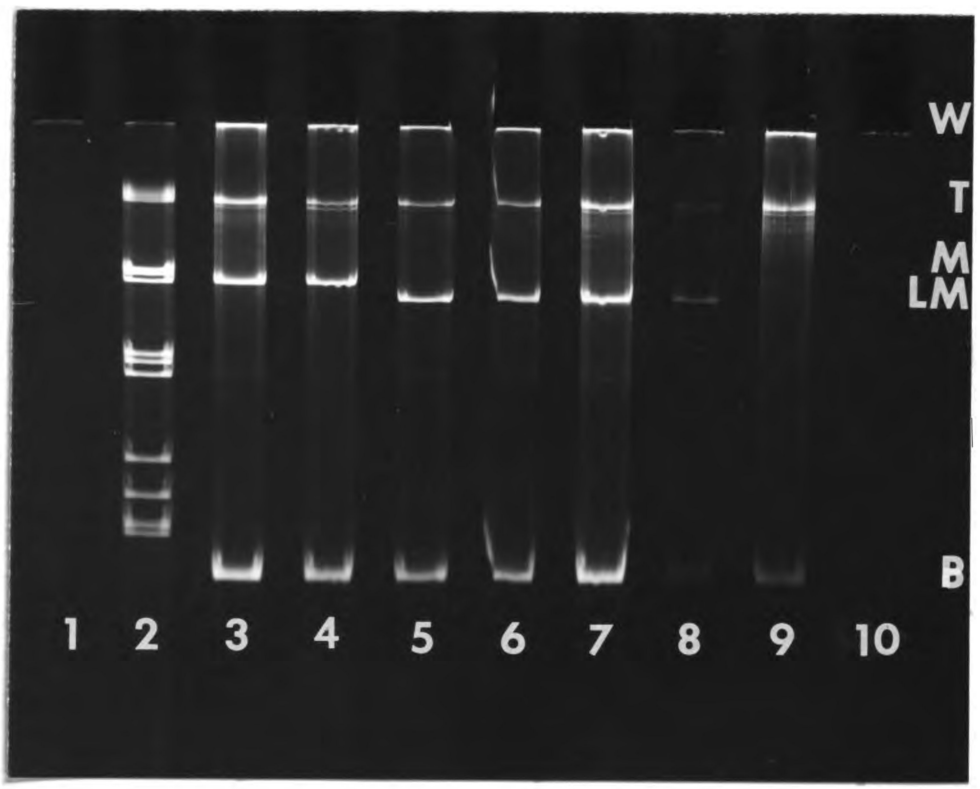


FIGURE 4

Table 9. Isolates obtained from cankers on the study trees at Crystal Lake, MI containing changes in the expected GH2 dsRNA banding pattern.

Pattern B: New band, lower than middle Tree (Canker position)	Date Isolated
T-G(R4-4)[1]	(5-83)
T-G(R1-3)	(7-83)
T-G(R4-4)	(7-83)
T-G(R5-4)	(9-83)
T-F(R3-5)	(9-83)
Pattern C: Lower middle band only **Tree (Canker position)**	
T-D(R2-3)	(5-83)
T-F(R3-5)	(7-83)
T-D(R2-3)	(7-83)
T-G(R4-2)	(9-83)
T-G(R4-4)	(9-83)
T-G(R1-3)	(9-83)
T-D(R2-3)	(9-83)
T-G(R5-1)	(9-83)
Pattern D: Missing middle band **Tree (Canker position)**	
T-G(R3-5)	(7-83)
T-F[2]	(9-83)
T-G(R3-5)	(9-83)

[1]Information enclosed in parenthesis after the tree letter indicates location of canker on the tree. The canker on this tree is located at the fourth wound site in the fourth row.

[2]If parenthesis do not follow the tree letter then the isolated culture originated from the third wound site of the third row for the indicated tree (not a canker).

Table 10. Chronological History of dsRNA banding patterns found in isolates containing changes in the expected GH2 dsRNA banding pattern.

	Type of dsRNA banding pattern observed					
	Date Sampled					
Isolated Culture	8/82	10/82	12/82	5/83	7/83	9/83
T-D(R2-3)[1]	-	-	A	C	C	C
T-F[2]	A	?	A	-	-	D
T-F(R3-5)	-	-	-	-	C	B
T-G(R1-3)	-	-	-	-	B	C
T-G(R3-5)	-	-	-	-	D	D
T-G(R4-2)	-	-	-	-	-	C
T-G(R4-4)	-	-	-	B	B	C
T-G(R5-1)	-	-	-	-	-	C
T-G(R5-4)	V	-	V	A	A	B

[1]Information enclosed in parenthesis after the tree letter indicates location of canker on the tree. The canker on this tree is located at the third wound site in the second row.

[2]If parenthesis do not follow the tree letter then the isolated culture originated from the third wound site of the third row for the indicated tree (not a canker).

A = CL1(GH2) PCNB banding pattern. Original GH2 banding pattern.

B = dsRNA banding pattern B, new band, lower than middle.

C = dsRNA banding pattern C, lower middle band only.

D = dsRNA banding pattern D, missing middle band.

V = Virulent, no dsRNA.

? = dsRNA banding pattern not determined because culture became contaminated.

- = No culture was isolated.

Table 11. Total number of cankers initiated by Endothia parasitica found on American chestnut trees in this study grouped in relationship to: A) The inoculum source placed on the tree; B) The phenotype of the isolated culture being the same as that of the inoculum source; C) The wound type made in the trees; and D) The distance the wound was located from the inoculum source.

A. Total number of cankers grouped by inoculum strain

Date	CL1	CL1 PCNB-R	CL1(GH2) PCNB-R	None	X^2
5/83	8	11	5	0	11.000*
7/83	12	13	12	6	2.860
9/83	18	25	17	12	4.778

B. Isolated culture same as inoculum source

Date	CL1	CL1 PCNB-R	CL1(GH2) PCNB-R	X^2
5/83	8	6	2	1.333
7/83	10	7	2	5.158
9/83	16	15	5	6.167*

C. Wound type

Date	cork borer	nail	scratch	slice	branch scar	X^2
5/83	5	18	0	0	1	48.916*
7/83	8	25	5	1	4	42.000*
9/83	17	32	10	6	7	32.027*

D. Wound distance from inoculum source

Date	10 cm	35 cm	60 cm	85 cm	110 cm	X^2
5/83	9	8	3	2	2	9.750*
7/83	12	12	10	6	3	7.349
9/83	20	19	12	14	7	7.861

Critical X^2 values for Inoculum Strain: P(0.10)=6.25, P(0.05)=7.81, P(0.025)=9.35, P(0.01)=11.3, P(0.005)=12.8.

Critical X^2 values for Isolated Culture Same as Inoculum Source: P(0.10)=4.61, P(0.05)=5.99, P(0.025)=7.38, P(0.01)=9.21, P(0.005)=10.6.

Table 11. (continued)

Critical X^2 values for Wound Type and Wound Distance: P(0.10)=7.78, P(0.05)=9.49, P(0.025)=11.1, P(0.01)=13.3, P(0.005)=14.9.

*Differences between strains, wound types or wound distances are significant at P=0.05 by X^2 test.

remained significant throughout 1983 (Table 11). No significant interactions between all combinations of the three factors (inoculum source, wound distance and wound type) were detected at any date (Table 12).

The average area of cankers calculated for the various strains isolated in September 1983 were 112.28 cm^2 for PCNB-S virulent strains, 59.45 cm^2 for PCNB-R virulent strains and 43.10 cm^2 and 70.00 cm^2 for PCNB-R and PCNB-S hypovirulent strains, respectively (Fig. 5). Average canker area at the different wound distances were 88.30 cm^2 , 74.30 cm^2 , 102.77 cm^2, 77.09 cm^2, and 125.38 cm^2 at 10, 35, 60, 85 and 110 cm distances, respectively (Fig. 6). Average canker area associated with the different wound types were 59.25 cm^2 for cork borer, 95.15 cm^2 for nail, 80.29 cm^2 for scratch, 81.19 cm^2 for slice and 146.57 cm^2 for branch scars (Fig. 7).

In May 1983, 8.33% of the cankers observed supported pycnidia, and by July 1983, 100% of the cankers supported pycnidia. Trees with confirmed hypovirulent cankers (positive dsRNA content as determined in the laboratory) contained approximately 75% of the sporulation observed in virulent cankers. In all cases, only pycnidia were observed (no perithecia).

Table 12. X^2 values for interactions due to inoculum source/wound distance, inoculum source/wound type and wound distance/wound type.

A. Inoculum Source/Wound Distance Interaction

Date	X^2
5/83	1.691
7/83	10.291
9/83	6.707

B. Inoculum Source/Wound Type Interaction

Date	X^2
5/83	2.691
7/83	16.288
9/83	12.954

C. Wound Distance/Wound Type Interaction

Date	X^2
5/83	5.406
7/83	18.620
9/83	13.772

Critical X^2 values for Inoculum Source/Wound Type and Innoculum Source/Wound Distance Interactions: P(0.10)=18.5, P(0.05)=21.0, P(0.025)=23.3, P(0.01)=26.2, P(0.005)=28.3.

Critical X^2 values for Wound Type/Wound Distance Interactions: P(0.10)=23.5, P(0.05)=26.3, P(0.025)=28.8, P(0.01)=32.0, P(0.005)=43.3.

Figure 5. Average canker area calculated by combining the various *E. parasitica* isolates based on their PCNB sensitivity and virulence status. PCNB-S/V = PCNB sensitive and virulent, PCNB-R/V = PCNB resistant and virulent, PCNB-R/H = PCNB resistant and hypovirulent, and PCNB-S/H = PCNB sensitive and hypovirulent.

FIGURE 5

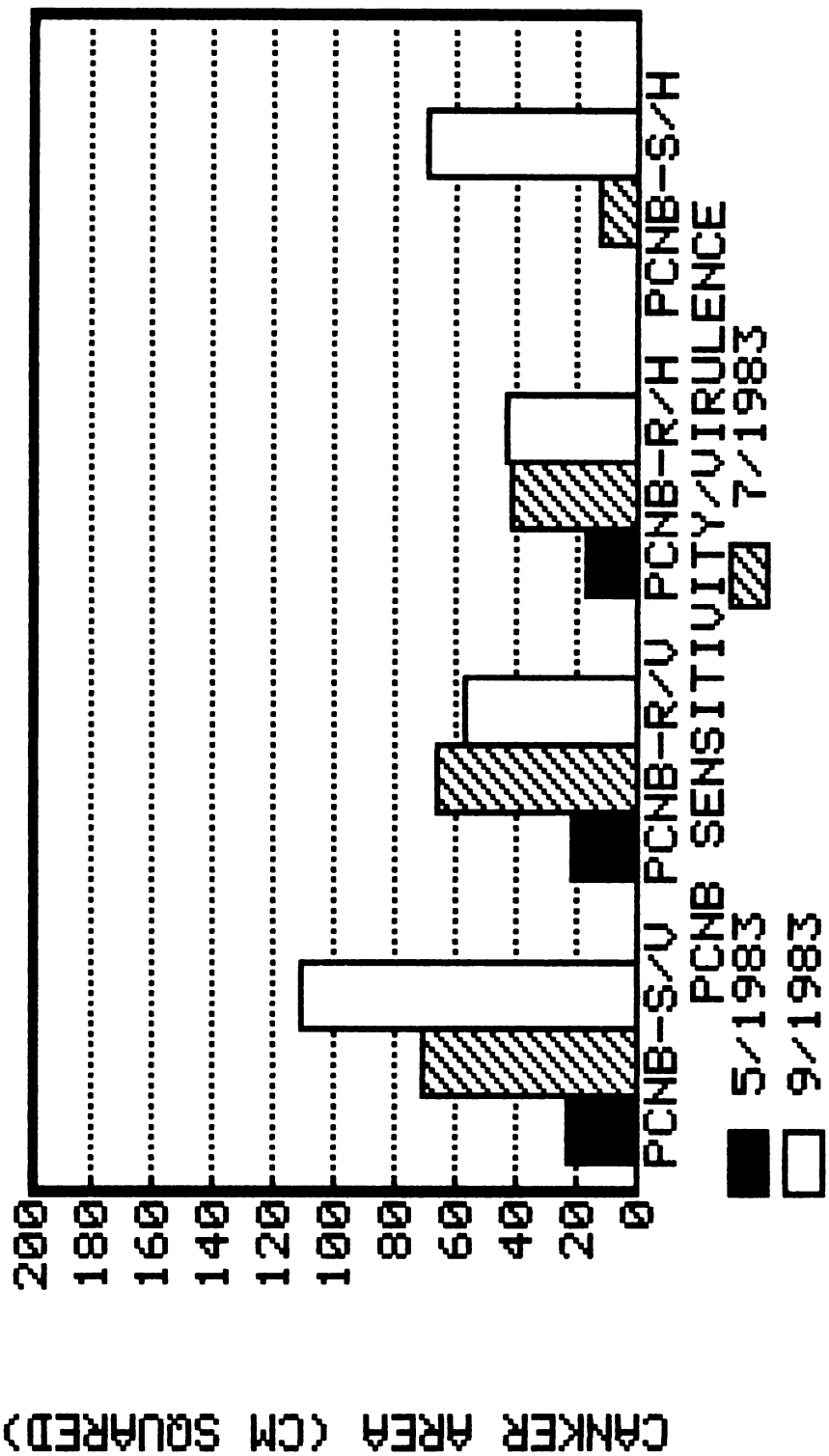

CANKER AREA (CM SQUARED)
200
180
160
140
120
100
80
60
40
20
0
PCNB-S/U PCNB-R/U PCNB-R/H PCNB-S/H
PCNB SENSITIVITY/VIRULENCE
5/1983
7/1983
9/1983

Figure 6. Average canker area associated with the various wound distances from inoculum sources.

FIGURE 6

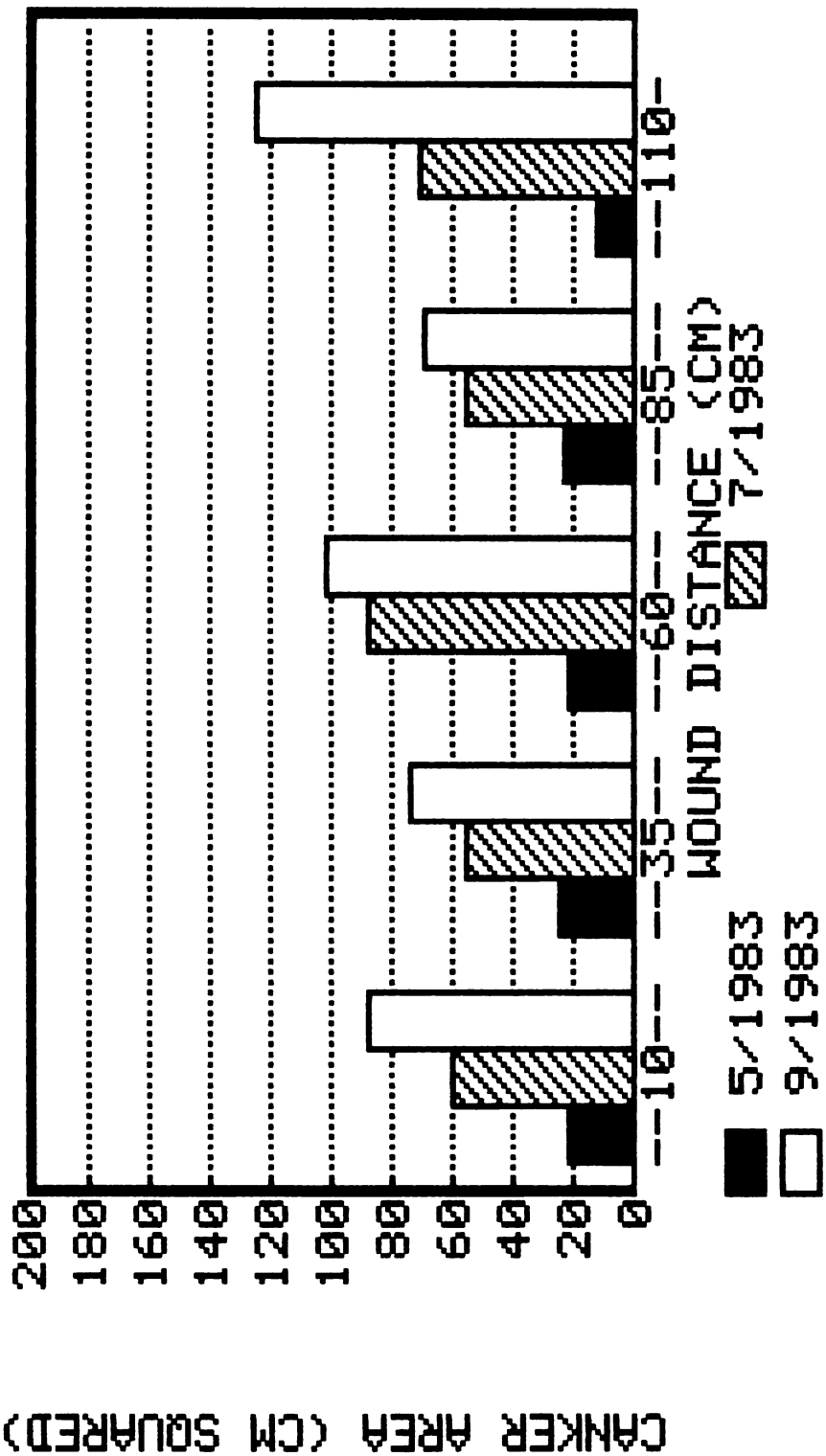
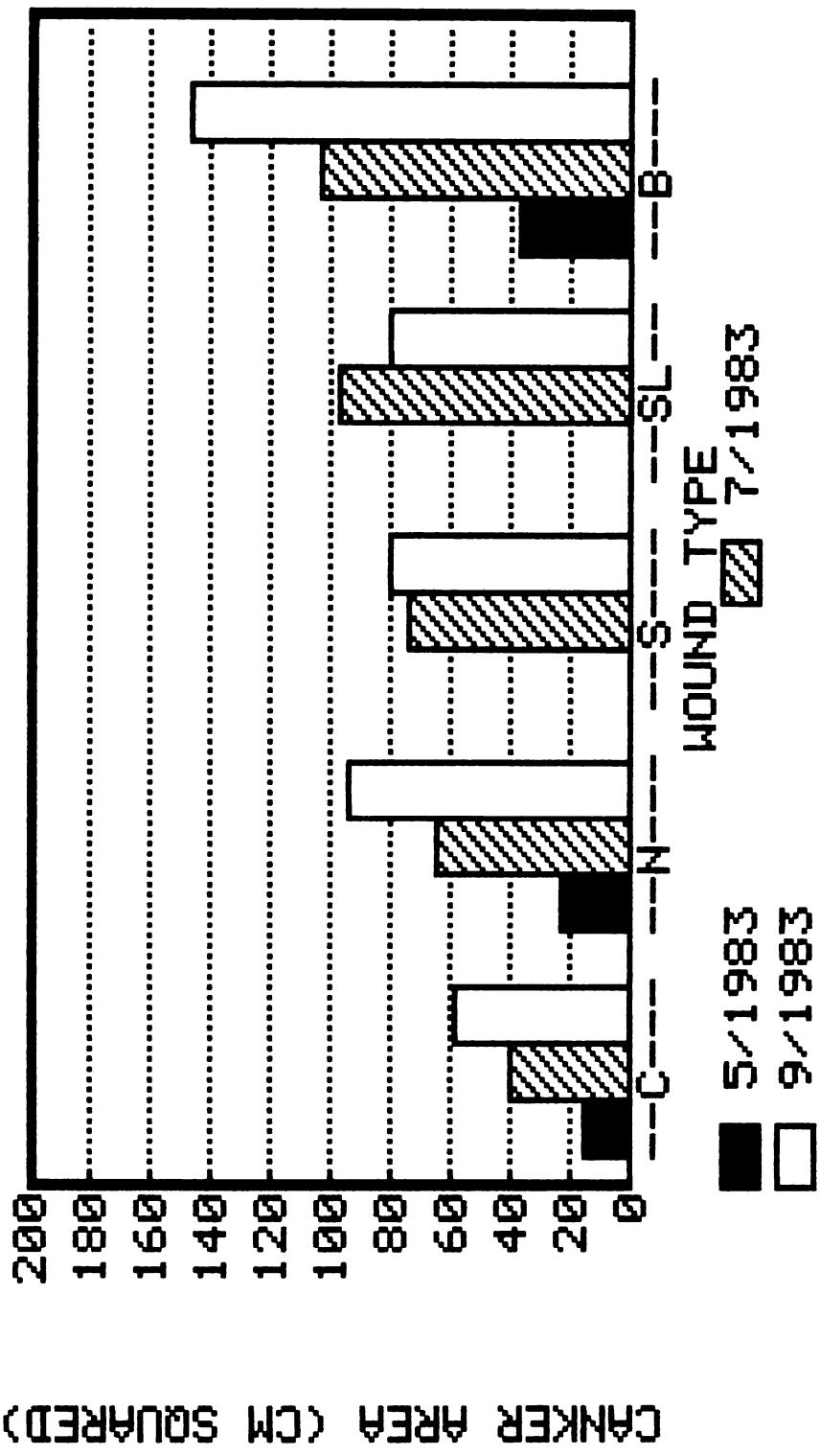

CANKER AREA (CM SQUARED)
200
180
160
140
120
100
80
60
40
20
0
10
35
60
85
110
WOUND DISTANCE (CM)
5/1983
7/1983
9/1983

Figure 7. Average canker area found associated with the various wound types. C = cork borer, N = nail, S = scratch, SL = slice, and B = branch scar.

FIGURE 7


CANKER AREA (CM SQUARED)
200
180
160
140
120
100
80
60
40
20
0
C
N
S
SL
B
WOUND TYPE
5/1983
9/1983
7/1983

DISCUSSION

The use of PCNB resistant strains in combination with the ability to identify characteristic dsRNA banding patterns of the hypovirulent inoculum source proved to be effective tools in monitoring the movement of *E. parasitica*. Spread of virulent and, more importantly, hypovirulent strains within and between trees in the grove was detected. In addition, there appears to have been natural conversion *in vivo* of virulent strains to hypovirulence by the passage of dsRNA from hypovirulent strains present on the same tree. This is the first experimental documentation of the natural spread and infection of a specific hypovirulent strain of *Endothia parasitica* in a blighted American chestnut grove in North America as documented by using specific dsRNA banding patterns and genetically marked strains.

Inoculum for the PCNB-R/virulent cultures isolated from trees with PCNB-R/virulent inoculum sources most likely originated from the PCNB-R virulent inoculum sources placed on the same trees. PCNB-S/virulent cultures obtained from trees with CL1 inoculum sources probably originated from either the CL1 inoculum sources placed on the trees or from the natural *E. parasitica* population.

The dsRNA banding pattern of the hypovirulent inoculum source appears to be very unique in that the bottom band (B) (Fig. 3) of the pattern has been found only in this particular hypovirulent isolate (38). Therefore, PCNB-R/hypovirulent cultures isolated from trees with hypovirulent inoculum sources most likely originated from the CL1(GH2) PCNB-R inoculum sources since these isolates contained dsRNA banding patterns identical, or very similar, to that of the hypovirulent inoculum source (Figs. 3 and 4).

Inoculum for PCNB-S/virulent cultures which were isolated from trees with PCNB-R/virulent and PCNB-R/hypovirulent sources of inocula (Table 8, isolate types one and two) could have originated from trees with CL1 inoculum sources, or from natural PCNB sensitive inoculum present in the grove.

The isolation of PCNB-R/virulent isolates from trees with PCNB-S/virulent and PCNB-R/hypovirulent inocula (Table 8, isolate types three and four) indicated that there appeared to be spread of PCNB-R/virulent inocula to these trees. Because prevailing winds are from the west, E. parasitica isolated from tree R may have originated from tree Q (Fig. 2). Apparently tree G, D or F was the tree from which inoculum for the canker on tree E originated. If it originated from tree D or F, the inoculum would have had to be a PCNB-R/hypovirulent strain which had lost its dsRNA or from conidia without dsRNA. It may be likely that this isolate was derived from a PCNB-R/hypovirulent strain

since a PCNB-R/hypovirulent isolate was collected from tree E on the same date. Tree A was presumably the source of inoculum for the isolate recovered from tree C. The PCNB-R/virulent isolate on tree M may have originated from the PCNB-R/hypovirulent inoculum source on tree M or from tree N. If from tree M, the inoculum would have had to be a PCNB-R/hypovirulent strain which had lost its dsRNA or from conidia without dsRNA.

PCNB-R/hypovirulent strains found on trees with CL1 PCNB-R and CL1 as inoculum sources (Table 8, isolate types five and six) probably originated from CL1(GH2) PCNB-R inoculum sources on nearby trees. Presumably tree T was the source of PCNB-R/hypovirulent inocula for the isolate obtained from tree G in December of 1982 (Fig. 2). Once established in tree G, it appears that hypovirulent inocula spread to other wounds, up and down the tree (probably by rain and insects), and was subsequently isolated upon several occasions. The canker found on tree E most likely originated from inoculum sources on trees F or D.

Because PCNB-S/hypovirulent _E. parasitica_ isolates were not introduced into the grove and because there is essentially no reversion to wild type by conidia from PCNB-R strains (D.W. Fulbright, personal communication) it appears that virulent PCNB-S mycelia present in wounds may have been converted to hypovirulence by hypovirulent strains also on the trees. Mycelium in canker E(R4-1) isolated in July 1983 (Table 8) presumably provided dsRNA for the conversion of natural PCNB-S mycelia which resulted

in the PCNB-S hypovirulent culture isolated in September of 1983 (canker E[R4-1], Table 8). The CL1(GH2) PCNB-R inoculum source located on tree F was most likely the source of dsRNA involved in the conversion of PCNB-S/virulent mycelia. Finally dsRNA found in a PCNB-S strain on tree G probably originated from other hypovirulent cankers on the same tree.

It is difficult to understand why PCNB-R virulent and hypovirulent inocula did not spread to trees without inoculum sources. It was suggested from infection studies that wounds on trees with inoculum sources are infected more quickly than are wounds on trees without inoculum sources (Table 11). The absence of inoculum sources on control trees may be one possible explanation of the failure to detect spread of PCNB-R virulent and hypovirulent inocula to these trees. Possibly the failure to observe movement of these particular strains was influenced by some unknown factor in the methods, or, it could have been due purely to chance. With further samplings from these trees, over time, dsRNA-containing strains may be detected on trees without inoculum sources. Other explanations for the lack of spread of PCNB-R virulent and hypovirulent strains to these trees may become apparent with further observations. However, these findings do not appear to detract from the detection of spread of hypovirulent strains to trees with virulent sources, because, hypovirulent isolates obtained from trees with virulent sources contained dsRNA banding patterns

identical, or very similar, to that of the hypovirulent inoculum source (Figs. 3 and 4).

Transmission of hypovirulence from strain to strain has been shown to occur by at least two methods: 1) to progeny through the production of conidia and 2) by hyphal anastomosis of compatible strains (85). Conidia have been shown to be disseminated by vectors including wind, rain, insects, birds, and small mammals (6, 19, 40, 49, 77, 82, 87). There is no reason to suspect that the dissemination of hypovirulent strains may not occur in the same manner. To substantiate this possibility, Fulbright (personal communication) reported the isolation of dsRNA infected conidia of E. parasitica from rain water dripping down an American chestnut tree trunk below a natural canker.

The observation that hypovirulent strains produce fewer pycnidia has been suggested as a possible barrier in achieving the natural dissemination of these strains (33, 85). For this reason, the CL1(GH2) PCNB-R strain was chosen for the experiment. This hypovirulent strain was selected because it sporulates well and is more virulent than other hypovirulent strains (it has the capicity to kill small suckers and seedlings when directly inoculated into them, but has not been shown to kill larger trees). Another desirable characteristic of this strain is that its dsRNA banding pattern is unique when compared to other dsRNA banding patterns of hypovirulent strains found, thus making the dsRNA easily recognizable when isolated on

polyacrylamide gels. Even though hypovirulent cankers in this study sporulated approximately 75% as much as virulent cankers, results obtained do not indicate that the production of fewer pycnidia by the hypovirulent inoculum source was an effective barrier in the prevention of the spread of the hypovirulent inocula. This statement is specifically supported by the spread of hypovirulence observed within tree G. It was dramaticlly demonstrated in this tree that once established, hypovirulent cankers can act as inoculum sources for subsequent hypovirulent infections.

Survival of hypovirulent strains among high densities of virulent inoculum has been discussed as a hinderance for the establishment of hypovirulent populations of _E. parasitica_ in heavily blighted chestnut groves (35, 85). This may be an explanation of the failure to recover hypovirulent strains on tree T (three sporulating virulent natural cankers were overlooked when this tree was selected for study). For hypovirulent strains to have an advantage over virulent strains it may be necessary to leave inoculum sources out for longer periods of time on trees which are heavily infected. Results on tree E however tend to contradict those found on tree T (tree E was also heavily covered with virulent cankers). Hypovirulence found on this tree, the first time, in July 1983 was apparently due to a hypovirulent strain from a nearby inoculum source and the second time, in September 1983 was the result of a natural conversion within the tree. The success of

hypovirulent strains on tree E as compared to tree T may be due to the possibility that virulent cankers on tree E were not as well established as those on tree T. Nevertheless, these data, along with data from tree F and tree G which also provide evidence of natural conversion, are promising in that they indicate that established virulent cankers may be converted to hypovirulence naturally.

The dsRNA segments of CL1(GH2), the hypovirulent strain from which the PCNB resistant strain used in the field experiment was isolated, have been transferred faithfully to numerous cultures derived from single conidial isolates of this strain (Fulbright, personal communication). It has also been shown (in European hypovirulent strains) that dsRNA banding patterns will remain intact through transfer (to virulent cultures) and upon subculture (5). Therefore it appears that the new dsRNA banding patterns detected in recovered field isolates were probably not due to incomplete transfer of dsRNA to conidia or through hyphal anastomosis.

When the chronological order of banding pattern appearance is analyzed (Table 10) it is noted that pattern D never preceeds A, B or C; pattern C does not preceed patterns A or B except in T-F(R3-5) and pattern B never preceeds pattern A. This suggests that in these experiments a shift has occured from pattern A to B to C to D. The hypovirulent culture obtained from the canker in tree F is the result of a natural conversion of virulent PCNB sensitive mycelium by dsRNA from the inoculum source

on the same tree. When this is taken into account, the continuity of the pattern sequence is preserved, thus explaining the appearence of pattern C before B in this particular situation.

An exclusion principle has been proposed to explain the loss of dsRNA segments found in Ustilago maydis , the corn smut pathogen (61). In this system it was found that certain dsRNA segments could not coexist in the same cell protoplasm, either one or both of the segments were lost from the fungal cells. Segments which were most affected were of medium and low molecular weights. In cases where a medium molecular weight segment was excluded from the cytoplasm, leaving the low molecular weight segment, it was hypothesized that the smaller molecules were retained because they had a replicative advantage.

A similar principle may be acting on the Endothia system, resulting in the observed alterations of the original dsRNA banding pattern of the hypovirulent inoculum source. All of the dsRNA banding patterns observed, whether changed or not, had the original top (T) and bottom (B) segments intact, thus providing evidence of the CL1(GH2) PCNB-R origin of the dsRNA. However, the middle segment (M) appeared to be unstable. Pattern B had an additional dsRNA segment (LM), which was slightly smaller than M; this segment may have been derived from M (M minus approximately 200 base pairs). For a period of time both bands could have existed together, then, possibly because the LM had a replicative advantage over M, M could have

been completely lost, giving rise to pattern C. Later, pattern D could have appeared when LM was lost. Because T and B were always retained, it indicates that they may have been packaged together and therefore always transferred together. Therefore, a possible explanation is that there may have been at least two virus-like particles (VLPs) infecting hypovirulent strains with the GH2 banding pattern.

Most natural cankers found on American chestnut trees at the Crystal Lake site were located primarily at wounds created by branch sites or scars (Garrod, unpublished). Wounds made by woodpeckers (which are similar to those made by nail and cork borer) were also seen, however, no signs of canker development were ever associated with these holes (Fulbright and Garrod, unpublished). The wound type versus infection results in this study were contrary to these observations. It was found that nail and cork borer holes, rather than branch scar wounds (whether artificial or natural) were more likely to have become infected.

Bazzigher (16) found that in older wounds, there was an increasing development of callus tissue, thus lessening the chance for infection. He stated, "if a wound parasite like _E. parasitica_ cannot enter the wounded tissue early enough, the infection fails or succeeds, but is greatly delayed." When branches die and break away from tree trunks a wound is created. These branch wounds expose all tissues of the tree, new and old (78). When small branches die, wounds close rapidly, but when large branches die, the

closing is slow (79). The mere physical structure of branch sites may be conducive to infection. The sites may trap and direct fungal spores into the crevices formed between the branch stub and tree trunk, thereby providing a direct pathway to susceptible tissues. Because certain branch sites are open to infection for a long period of time, this could explain why there are so many naturally existing cankers at these positions on American chestnut trees. Woodpecker wounds however, could close much more rapidly than branch site wounds and may therefore have a shorter period of susceptibility to infection. In this study, inoculum was provided to all wound types at the same time, most likely before callus tissues could form in the wounds. The data suggested that when exposed to inoculum, before wound closing could take place, nail and cork borer holes are more efficient (in becoming infected) than were other wounds tested. If this experiment were to be continued, based upon the conclusions drawn above, it would be expected that, ultimately, an increasing number of cankers would appear at natural branch scar wound positions (because they may not close quickly) and that the number of new cankers at the nail and cork borer sites would diminish.

Support for the ideas presented above is provided by average canker area data. Cankers located at branch scars had the largest average area of all other wounds, indicating that these wounds (branch sites and scars) are most likely to be successful if infected (success being

defined in terms of area). The average area of cankers located at slice wounds in September 1983 decreased due to the failure to reisolate E. parasitica from existing cankers.

Wounds at all distances were found to be equally infected. Therefore, it has been shown that 1) conidia can be carried in some manner 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 2) the susceptibility of the host plant is homogeneous within the distance range of the wounds on the trunk.

Bazzigher and Schmid (18) found that the susceptibility to infection of four-year-old chestnut plants differed within a range of 20 cm on the trunk. Upper wounds were less likely to become infected than were lower ones. In addition, the average length of cankers at the upper wound spots were only half as large as were those at lower positions. They believed that the reason for this variation in susceptibility depended upon different healing abilities of the tissue in different parts of the tree. It must be noted that the trees used by Bazzigher and Schmid were much younger than those used in this study, and, therefore, tissues 20 cm apart in younger trees would be more variable than would those the same distance apart in older trees (such as those used in this study). This could explain why they found differences in the number of infections 20 cm apart and I did not. However, data

obtained in this study and that by Bazzigher and Schmid do agree on the observations that lower cankers were larger than those found further up the tree. For reasons stated above in regards to tissue differences over distances in younger versus older trees, an alternate explanation for these data may be more reasonable. Perhaps cankers at lower positions were larger and more numerous, in their study and this one, because lower tissues were being deprived of nutrients due to the girdling effect of higher cankers. If this were true, lower cankers would be larger because tissues below upper cankers would be weaker, and therefore less able to defend against fungal invasion.

According to data collected in May 1983, wounds on trees that had inoculum sources were more likely to become infected than were wounds on trees that did not have inoculum sources. These data show that wounds can become readily infected if inoculum sources are available on trees. Later in the study, data indicated otherwise. At the July and September 1983 sampling dates, essentially the same number of cankers were found on all trees regardless of the previous presence of an inoculum source. If the data are examined (Table 11) it becomes apparent that the number of cankers increased equally on all trees. These new cankers were initiated due to the spread of natural inoculum that was equally available to all study trees. The continuing increase of cankers masked differences that had previously existed. If inoculum sources were continually present during the entire study, from June 1982

until September 1983 (rather than from June 1982 until December 1982), then it is likely that there may have been a larger number of cankers on trees with inoculum sources than on trees without.

Areas of cankers were compared according to the strain type that was isolated from them. Canker area has been used in other studies as a measure of virulence (33, 38, 86); strains which are more virulent produced larger canker areas. It was found that virulent PCNB-S cultures were isolated from cankers which were larger than those from which virulent PCNB-R were isolated. This was expected because it has been shown that PCNB-R strains are less virulent than sensitive strains (Fulbright, personal communication). The average canker area produced by virulent PCNB-R strains decreased in September of 1983 because some of these cankers were converted to hypovirulence and were therefore calculated with the hypovirulent PCNB-R group. Canker areas in which hypovirulent PCNB-S strains were isolated increased sharply in September 1983 because these cankers were originally initiated by virulent strains. It would be expected that the areas of these cankers would not increase as rapidly as they had because the cankers are now infected with hypovirulent E. parasitica .

There were no significant interactions between factors determined significant. This indicates that a particular inoculum source strain does not prefer a particular wound type at a specific wound distance.

Data obtained from this study may have useful applications in achieving the natural dissemination of hypovirulent _Endothia parasitica_ . Control of virulent cankers may be more rapidly and effectively reached with use of hypovirulent inoculum sources, rather than the cork borer, inoculum plug procedure (10). However, more research must be done to determine the practicality of this method.

PART II

A. THE DETERMINATION OF MATING TYPES OF AMERICAN VIRULENT AND HYPOVIRULENT STRAINS OF ENDOTHIA PARASITICA .

B. THE SEARCH FOR dsRNA IN SINGLE ASCOSPORE ISOLATES OF ENDOTHIA PARASITICA OBTAINED FROM (1) SEXUAL CROSSES INVOLVING HYPOVIRULENT STRAINS AND (2) PERITHECIA REMOVED FROM CANKERS WHICH WERE TREATED WITH HYPOVIRULENT ISOLATES.

INTRODUCTION

Perithecia are rarely formed by European hypovirulent strains in both controlled laboratory crosses and in hypovirulent and/or hypovirulent-treated cankers (35, 58, 85). In the few cases in which perithecia are obtained from these interactions, transmission of dsRNA to the ascospores has not been observed (2, 30, 85).

Because the ascospores are probably the long distance dispersal units of the fungus (2, 12, 13, 50, 51), it would be most advantageous to find dsRNA in these spores to facilitate the spread of hypovirulence.

Anagnostakis has identified mating type testers which may be used in effecting controlled laboratory crosses of Endothia parasitica . One mating type locus with two alleles (A,a) has been found so far (4).

The objectives of this research were to (a) determine the mating types (using established testers) of a number of Endothia parasitica isolates collected from American chestnut groves located in Michigan including the three inoculum source strains used in part I of this thesis, and to (b) detect dsRNA in ascospores isolated from perithecia formed by the crosses above and from virulent cankers which had been treated with American hypovirulent strains.

MATERIALS AND METHODS

Isolates used

Thirty-five isolates of *Endothia parasitica* collected from American chestnut groves located at Crystal Lake and Grand Haven, Michigan and the three inoculum source strains used in part I of this thesis (CL1, CL1 PCNB-R ,and CL1(GH2) PCNB-R) were paired with themselves, known mating types (EP110 A and EP 329 a or EP 42 A and EP 339 a, obtained from S. L. Anagnostakis [The Connecticut Agricultural Experimental Station, New Haven, CT]) and each other (in some cases). Field isolates analyzed were single conidial, single ascospore and mass isolate bark cultures of virulent and hypovirulent strains.

Sexual matings

Ascospores were obtained from perithecia from crosses which involved hypovirulent strains. Representative single ascospore cultures with hypovirulent-like morphologies were analyzed for the presence of dsRNA according to the method of Day et al. (30) with modifications by Dodds (31) and Fulbright (38). In addition, ascospore cultures obtained

from the cross between CL1(GH2) PCNB-R and EP 110 A were tested for resistance to pentachloronitrobenzene by subculturing ascospore cultures onto ECA (71) with 100 µg PCNB/ml. Control crosses between complementary mating types accompianied each sexual mating trial.

Bark samples supporting perithecia were collected from naturally and artificially inoculated cankers on fourteen trees at the Crystal Lake site. Most cankers had been treated with hypovirulent strains (see Table 1). Five ascospores were randomly selected from each of five perithecia that were isolated from each sample. Selected single ascospore cultures with hypovirulent-like morphologies were analyzed for the presence of dsRNA (using methods described in part I of this thesis).

In vitro matings of E. parasitica were carried out by the methods of Anagnostakis (3) except where modified as below.

Sections of healthy chestnut stems, approximately 6 cm x 1 cm x 0.7 cm were autoclaved twice at 15 psi for 30 minutes. The stems were transferred aseptically to 100 x 15 mm plastic petri plates and orientated so that the bark surface was facing the top of the plate. Four percent water agar (Difco; Detroit, MI), without biotin and methionine, was autoclaved at 15 psi for 20 minutes, cooled to approximately 40 C, and gently poured around the chestnut stems to a depth which covered half the branch.

Inoculum was grown on potato dextrose agar (PDA; Difco; Detroit, MI), which was not amended with biotin and

Table 1. Histories of cankers on American chestnut trees from which bark samples were collected in June 1982.

Tree-canker number	canker origin[1]	canker treated[2]	control achieved[3]
5-2	artificial	yes	?
5-8	artificial	yes	yes
6-1	artificial	yes	no
11-1	artificial	no	-
12-1	natural	yes	yes
13-2	natural	yes	no
14-3	artificial	yes	no
17-1	natural	yes	yes
18-2	natural	yes	no
20-1	natural	yes	yes
21-1	natural	yes	yes
21-2	natural	yes	yes
24-6	natural	yes	yes
24-1	natural	no	-
26-1	natural	yes	yes

[1]Artificial cankers were initiated in June 1981 by filling a 7 mm diameter cork borer hole with a disc of virulent *E. parasitica* mycelium, and the PDA it was growing on. Inoculated wounds were covered with masking tape to prevent dessication of the inoculum.

[2]Cankers were treated in July 1981 by filling 7 mm diameter cork borer holes placed around the periphery of established cankers with discs of hypovirulent *E. parasitica* mycelium, and the PDA they were growing on. Inoculated wounds were covered with masking tape to prevent dessication of the inocula.

[3]Biological control of the treated cankers was considered achieved if expansion of the cankers was prevented.

methionine. Seven mm diameter agar discs, cut from 5 to 10 day-old inoculum cultures, were placed on either side of the chestnut stems. Petri plate perimeters were sealed with parafilm to prevent contamination. Plates were incubated at approximately 24 C under fluorescent lights with a 16-hr photoperiod.

Ten to 20 days later, when conidia were issuing from pycnidia, 3 ml sterile distilled H_2O was added to each plate. Conidia were spread over the stem surface by shaking the plate vigorously for 30 seconds. Cultures were incubated as described above.

Plates were checked every week for 16 weeks under a dissecting microscope for signs of perithecial development (usually 2-8 weeks).

Ascospore isolation

Ascospores were removed from perithecia and isolated by the methods of Puhalla and Anagnostakis (71) except where modified as follows.

Perithecia were aseptically dissected from the chestnut bark with sterile tweezers. Individual perithecia were macerated in a sterile test tube with 1 ml sterile distilled water, vortexed for 1 minute to facilitate ascospore release, and the resulting suspension was distributed over 4% water agar, 0.1 ml per plate. Cultures were incubated 24 hours at approximately 24 C under fluorescent lights with a 16-hr photoperiod.

Germinated ascospores were identified at 45X magnification with a dissecting microscope. Ascospores were distinguished from conidial contaminants by the presence of two germ tubes emerging from each cell of the ascospore (48). Small pieces of agar, each containing a single ascospore, were cut out and transferred to PDA, five spores per plate. Cultures were incubated as described above. Three to five days later, isolates were subcultured onto fresh PDA. Morphologies of the single ascospore colonies were rated according to mycelial appearance and growth rate after seven days.

RESULTS

Mating types were assigned to 22 of the 35 isolates tested. Seventeen were type A, five were type a and one isolate formed perithecia when crossed with itself (Table 2). The remaining 12 field isolates did not form perithecia. Seventeen of the 22 successful crosses included hypovirulent strains. One of the seventeen was a cross which was made between two hypovirulent strains (GH2 and GHU4). This particular mating was successfully repeated four times (Fig. 1). Some single ascospore cultures from the cross involving GH2 and GHU4 had unusual culture morphologies on PDA which were typical of GHU4, the more debilitated of the two hypovirulent strains. Nine of these abnormal cultures from five separate perithecia were analyzed for dsRNA upon two separate occasions. In all cases dsRNA was not found to be present.

Mating types of the three inoculum sources were all type a. Seven morpholigically abnormal single ascospore cultures from two perithecia obtained from the CL1(GH2) PCNB-R x EP 110 A cross were assayed for dsRNA. Again, dsRNA was not found. Seventy-three single ascospore cultures from the same two perithecia were tested for PCNB resistance. Forty-three were resistant and 30 were not. A

Table 2. Mating types of *Endothia parasitica* strains involved in successful sexual crosses.

Strain	Location	Isolate Type[1]	Mating Type
GH2	Grand Haven	H, mass	A
GH2B2	Grand Haven	H, single C	A
GH2F3	Grand Haven	H, single C	A
GH2K5	Grand Haven	H, single C	A
GH2L6	Grand Haven	H, single C	A
GH2N1	Grand Haven	H, single C	A
GH21B	Grand Haven	H, mass	A
GHA	Grand Haven	H, mass	-
GHU2	Grand Haven	H, mass	A
GHU3	Grand Haven	H, mass	A
GHU4	Grand Haven	H, mass	a
GH6	Grand Haven	H, mass	A
GH7	Grand Haven	H, mass	A
GH8?	Grand Haven	H, mass	A
GH14	Grand Haven	H, mass	A
TAc1MSSI	Grand Haven	H, mass	A
TAc2MSSI	Grand Haven	H, mass	a
CL1	Crystal Lake	V, mass	a
CL1-16	Crystal Lake	V, single C	a
CL4	Crystal Lake	V, mass	A
CL25	Crystal Lake	V, mass	A
T12SS2	Crystal Lake	V, single As	a
T12SS5	Crystal Lake	V, single As	A

[1]Mass isolates of *E. parasitica* were obtained by subculturing mycelia which were growing on PDA from bark samples that were taken from cankers on American chestnut trees.

H = hypovirulent
V = virulent
C = conidial
As = ascospore
- = isolate formed perithecia with itself

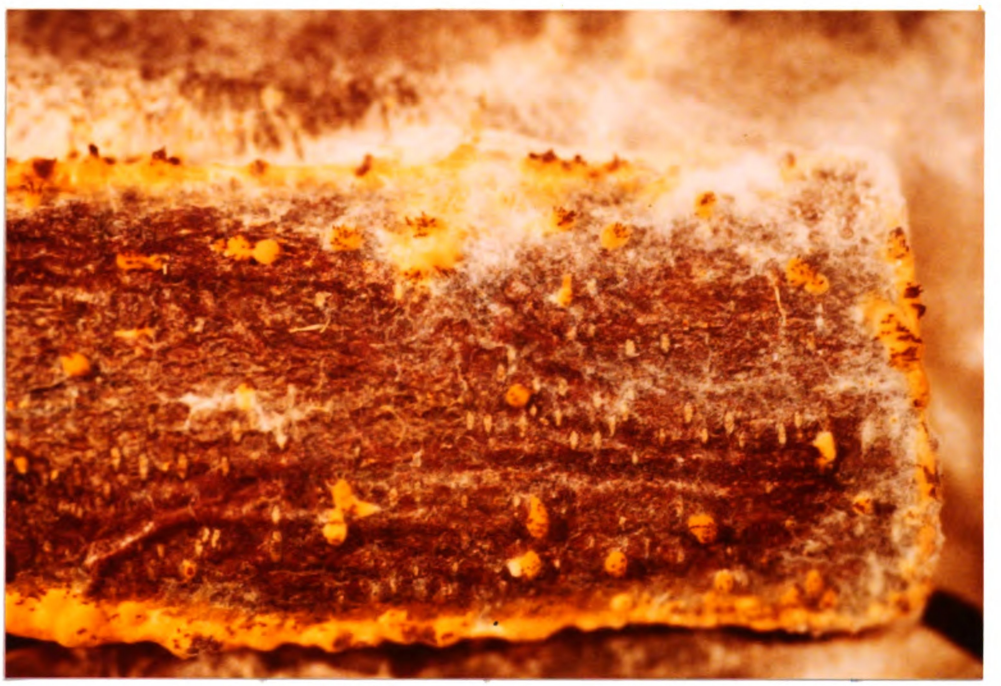


Figure 1. Perithecia formed by the cross of two hypovirulent strains, GH2 and GHU4.

X^2 analysis (81) at P=0.05 found no significant difference between these two values.

Abnormal morphology was also associated with a number of the 300+ single ascospore cultures obtained from the perithecia removed from field-bark samples. Six of the cultures from a total of three perithecia were tested for the presence of dsRNA (three of the isolates were checked twice). dsRNA was not observed in any of the cultures.

DISCUSSION

Puhalla and Anagnostakis (71) found that perithecia of Endothia parasitica could arise from the sexual fusion of two genetically identical or different nuclei. This suggested that the fungus was homothallic, but could outcross preferentially. Evidence supporting these conclusions was obtained from the present and other studies (1, 4). Subsequent investigations have shown that the ascogenous nuclei of E. parasitica can come from at least three parents, one female and two males, thereby further increasing the potential for genetic diversity provided by sexual propagation (8).

The non-significant X^2 value obtained in the statistical analysis of PCNB resistance in progeny from the CL1(GH2) PCNB-R, EP 110 A cross implies that resistance to PCNB is carried on the fungal nuclear genome.

Mating methods described by Anagnostakis (3) proved to be successful in 64 percent of the crosses performed. Failure of the remaining matings to produce perithecia may be partially attributed to (a) contamination of the chestnut stems by Penicillium sp., which almost always occured over the long incubation periods required by the procedure and/or (b) the ability of some strains to

function only as males (4).

Hypovirulent strains involved in crosses were found to be repeatedly successful in forming perithecia, contrary to results reported by others (35, 85). Hypovirulent strains used by others were primarily those obtained from European and/or derivitives of European strains. This factor may account at least partially for the observed differential abilities of hypovirulent strains to form perithecia. However, in this study as well as in others (30), dsRNA was not found to be incorporated into the ascospores. This phenomenon was observed in both single ascospore cultures that were derived from crosses that involved one and two hypovirulent strains and in those cultures that were obtained from ascospores that were isolated from perithecia which were present in hypovirulent cankers. Day et al. (30) suggested that transmission of dsRNA through ascospores derived from perithecia produced in healing cankers was not observed because the perithecia were collected from the central, older region of the canker which may have been formed by islands of virulent mycelia. This may be true, nevertheless, because dsRNA was never found in controlled laboratory crosses involving hypovirulent strains, it may be stated with confidence that transmission of dsRNA to the sexual spores of Endothia parasitica is very rare or does not occur.

Gaeumannomyces graminis , the fungal pathogen responsible for the take-all diseases of cereals can be infected by virus-like particles (VLPs) (76). VLPs in G.

graminis are transmitted inefficiently into the ascospores (75). It has been suggested that because VLPs can pass from mycelium into basidiospores (in Ustilago sp. infected with VLPs) and conidia, a physical barrier preventing entry into ascospores is unlikely (80). However, because ascogenous hyphae grow very quickly, like hyphal tips, and are quickly delimitated by septa (27), VLPs may not have sufficient time to enter the developing asci (76). It has also been suggested that the ascospore environment could be chemically unsuitable for virus-like particle inhabitation, possibly due to an undesirable pH (76). Some, all or none of these suggestions may be responsible for the apparent exclusion of dsRNA from ascospores of E. parasitica .

Hypovirulence in E. parasitica is found in a number of locations, great distances apart (38). Because dsRNA has not been found to be incorporated into the ascospores (the long distance disseminators of the fungus) it appears that hypovirulence is not spread by these spores. The question of the origin of dsRNA has remained unanswered. It has been suggested that it was introduced into E. parasitica by a closely related fungus, Endothia radicalis (Schw.) DeNot. (35) or possibly by a unrelated species or species' of fungi or other microorganisms (W. H. Weidlich, personnal communication). It has also been suggested that a proviral stage may be involved and that copies of the dsRNA are integrated into the fungal chromosome as DNA (85). No positive evidence for any of these hypotheses are presently available.

APPENDIX

APPENDIX

ADDITIONAL FIELD EXPERIMENTS

Two additional field experiments were carried out at the Crystal Lake site. The first was designed to study the infection of the American chestnut by Endothia parasitica based upon strain, wound type and time of inoculation after wounding. E. parasitica strains and wound types were the same as described in part I of this thesis. Eight sets of wounds were made in a randomized order on two trees. Four of the wound series were inoculated immediately after wounding and the remaining four were inoculated 24 hours later. Inoculations were made by directly placing a small disc of fungal mycelium (growing on PDA) into the wounds. Inoculation sites were covered with masking tape to prevent dessication of the inoculum. Uninoculated wounds were not covered.

Infections took place in all wound types that were inoculated at both times. Uninoculated wounds were infected equally well. These data indicate that the inocula used could cause infection in wounds of these types up to at least 24 hours after the wounding had occured.

The second field experiment involved a descriptive

study of existing natural cankers. Areas of 11 cankers on 7 trees were calculated four times over 14 months. The presence of pycnidia and perithecia in the cankers were also recorded as either "+" fruiting bodies present or "-" fruiting bodies absent.

Data indicated that the average canker increased approximately 2.5 times its original area over the 14 month time span. Pycnidia were observed in all cankers at each observation. Perithecia were observed in 18% of the cankers at the initial observation and in 100% of the cankers at the second observation (one month later) and in all remaining observations.

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