Natural Infection of Nuts of *Castanea dentata* by *Endothia parasitica*

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ABSTRACT

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An average of 14% of the nuts harvested from a planting of American chestnuts in which chestnut blight was prevalent was infected with *Endothia parasitica*. The percentage of nuts infected from individual trees varied greatly, but samples from 32 of 37 trees had one or more infected nuts. Signs of infections by the pathogen appeared after storage at 4 C followed by incubation at 18–25 C, but the infections apparently were initiated while the nuts were on the tree. Infections were confined to the shell and appeared not to effect seed germination or seedling growth; pycnidia were commonly produced. Nuts of other chestnut species and hybrids from the same location appeared not to be infected. Immersion of nuts in water at 50 C for 30 min shortly after harvest diminished, but did not eradicate, *E. parasitica*. Transportation of nuts of *C. dentata* grown in areas where *E. parasitica* is present could be a means of transmitting the pathogen long distances to areas presently free of the disease.

Additional key words: hypovirulence, seedborne pathogen.

The chestnut blight fungus, *Endothia parasitica* (Murr.) P. J. and H. W. Anderson, is normally disseminated by ascospores and conidia and, perhaps, by mycelial fragments. Long distance transport may result from the physical movement of infected stems and subsequent dispersal of spores and mycelium. We and others (9) have assumed that infection arising from nuts would be initiated by surface contamination with the pathogen, which could be eliminated by surface disinfection. However, more than a half century ago, Collins (1,2) found infected chestnut fruit in the fall at the time of ripening. The tree species, identified only in the second paper, was *Castanea sativa* Mill., the European chestnut. Only one report of infections on American chestnut fruit, *C. dentata* (Marsh.) Borkh., was found, and that was a brief reference by Gravatt et al (6) in 1953 to one lot of American chestnuts (from North Carolina, number of fruit not stated) in which 23% were infected with the blight fungus. These authors found no infections on many thousands of chestnuts imported from the Orient. We observed apparent signs of infection by *E. parasitica* on germinating nuts of *C. dentata* in February 1981 while bench-grafting chestnut (3). The present study was undertaken to determine how frequently this occurred, whether the chestnut blight pathogen could be readily spread by infected nuts, whether heat treatment could eradicate the pathogen from infected nuts, and whether the nuts were infected with hypovirulent or normally virulent strains.

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MATERIALS AND METHODS

Nuts were harvested predominantly from a planting of 14- to 16-yr-old trees at Lockwood Farm, Hamden, CT, in late September 1980, 1981, and 1982. Over 100 American chestnut trees were growing on the 0.5-ha area, and naturally occurring chestnut blight infections were common. About half of the trees were bearing fruit (nuts), and all of these had one or more cankers. Cankers on many trees had been inoculated with mixtures of hypovirulent strains in an attempt to maintain the trees (7). Some nuts were collected from the ground the first year. In subsequent years, burrs were picked before nut release to avoid possible contamination with soilborne organisms. The burrs of each tree were held in separate wire crates in a cool, humid cellar for 1 wk to allow complete maturation, and then the nuts were extracted. Nuts from each tree were kept separate and stored in Canadian peat moss in plastic bags, generally at 4 C until removed 11 or more weeks later. Nuts from five trees in 1981 were also stored at 13 and 21 C to compare the effects of temperature on the development of infection.

Shortly after harvest in 1982 a portion of the nuts from nine trees

was heat treated by immersion in water at 50 C for 30 min to determine if this would inhibit development of *E. parasitica*. This treatment is used to kill weevils in nuts (10). The effect of heat on mycelium and conidia of two morphologically normal, laboratory-grown cultures of *E. parasitica* was also tested. Mycelium-agar plugs (6-day-old cultures), mycelium with conidia-agar plugs (24-day-old cultures), and conidial suspensions were heat treated in vials held in a water bath at 40–60 C for 20–30 min and subsequently tested for viability on potato-dextrose agar (PDA) plates at 23 C.

After storage, nuts were sown in flats of moist peat moss and in a greenhouse at 18–25 C. They were examined for mycelial fans and pycnidia after 4–9 wk. Samples of nuts with apparent signs of infection were used to isolate the fungus in culture. These cultures were compared to stock cultures of *E. parasitica* under standard conditions (11). Twenty-six of our first isolates (three replicates each) were inoculated into American chestnut sprouts in May 1981, and measurements of cankers were made 4 mo later. Two dsRNA extractions (4,8) were run on 14 isolates having the most abnormal cultural morphology. The presence of dsRNA is indicative of viruslike particles and also is characteristic of hypovirulent (blight curing) strains of *E. parasitica,* as is abnormal cultural morphology (5).

Several hundred nuts from Chinese (*C. mollissima* Bl.), European (*C. sativa*), Japanese (*C. crenata* Sieb. & Zuc.), and hybrid chestnut trees at the same and other locations were also examined for the presence of *E. parasitica*. Seedlings grown from American nuts with and without apparent infections caused by *E. parasitica* were field planted after germination and observed for up to 2 yr to determine if there were differences in growth rate or percent of trees infected.

RESULTS

Examination of American chestnuts collected in the fall of 1980, stratified, and subsequently incubated for 4–6 wk in the greenhouse, indicated that approximately 31% were infected with *E. parasitica* based on the presence of mycelial fans and/or fruiting bodies (pycnidia) (Table 1). The characteristically orange-colored fans were thin and apparently confined to just below the epidermis of the nut shell. No evidence was noted of penetration into the pellicle or embryo. Fruiting bodies erupted through the shell and were located on the smooth shell surface or the textured hilum (Fig. 1). Twenty-six of these nuts with fruiting bodies were sampled and fungal isolates resembling *E. parasitica* were obtained from all of them. No *E. parasitica* was recovered from 15 nuts with only mycelial fans. Contamination with other organisms was a problem in isolations attempted from nuts with only mycelial fans. However, two nuts with mycelial fans later produced fruiting bodies, and fungal isolates resembling *E. parasitica* were recovered from them. For data reported for the 2 subsequent years, only nuts that produced pcynidia were classified as infected.

Over 5,400 nuts from 37 American chestnut trees were examined from the 1981 and 1982 harvests, and 11–14% of these were infected (Table 1). These percentages are conservative, because it is likely that some of the nuts showing only mycelial fans also were infected. Thirty-two of the 37 trees produced one or more infected nuts, and 10 of 11 trees, from which nuts were harvested both years, had infected nuts both years. Isolations of cultures resembling *E. parasitica* were made from over 264 nuts from 17 trees. Nuts with pycnidia invariably yielded cultures that resembled *E. parasitica*. Of all the nuts examined over a 3-yr period, only two were found on which pycnidia had developed at the time of harvest.

Double-stranded RNA extractions were run on 14 isolates with apparent abnormal cultural morphology; no dsRNA was detected. All 26 of the isolates resembling *E. parasitica* inoculated into sprouts appeared to be pathologically normal *E. parasitica*.

Approximately 50 seedlings each from infected and uninfected nuts were grown each year in nursery rows in the field. Blight infections on the 1- and 2-yr-old plants were rare (less than 2%) and apparently not related to infection of the nut shell.

Nuts of other chestnut species and hybrids were also collected from trees at the Lockwood Farm and were stored and incubated under the same conditions as those used for the American chestnut nuts. These were: *C. mollisima* (two selections, 612 nuts), *C.*

TABLE 1. Number of nuts collected from American chestnut trees in Hamden, CT, and percent infected with *Endothia parasitica* for each of 3 yr

Harvest year	Total nuts (no.)	Incubation period after storage (wk)	Infected[a] (%)
1980	234	4–6	31.2
1981	3,364	8	14.1
1982	2,069	4	11.4
Totals	5,667		13.8

[a] Infection was determined by the presence of mycelial fans and pycnidia in 1980, but only by the presence of pycnidia in the subsequent 2 yr.

Fig. 1. Signs of infections caused by *Endothia parasitica* on American chestnuts. *Left,* orange mycelial fan just below the epidermis, and *right*, pycnidia.

TABLE 2. The effect of heat treating American chestnut nuts in the fall at 50 C for 30 min on subsequent development of pycnidia of *Endothia parasitica* in the shells

Tree	Untreated		Heat treated	
	No. nuts	Infected (%)	No. nuts	Infected (%)
R2T1	113	.09	50	0
R2T3	132	6.1	38	0
R3T7	30	26.7	34	0
R4T1	111	27.0	64	1.6
R4T3	66	21.2	58	0
R4T6	51	17.6	55	1.8
R4T9	44	45.4	49	0
R4T10	45	68.9	43	0
RSTC	66	31.8	57	0
Total	658	21.6	448	0.4

TABLE 3. The effect of 40–60 C for 20–30 min on the viability of mycelium and conidia of *Endothia parasitica* subsequently plated on PDA

Temp [a] (C)	6-Day-old mycelium in PDA		24-Day-old mycelium and conidia in PDA		Conidial suspension in H_2O	
	Samples [b]	Growth [c]	Samples	Growth	Samples	Growth
40	10/10	+	10/10	+	10/10	+
50	30/30†	+–	30/30	+	18/30†	+–
53	0/10	–	10/10	+	8/10†	+–
55	1/10	–+	8/10	+–	0/10	–
56	0/10	–	3/10	–+	7/10†	+–
60	0/20	–	5/20	–+	1/20	–+

[a] Exposure in water bath for 30 min except for 53 and 56 C samples, which were treated for 20 min.
[b] Number of samples (different plates) that grew over total number tested. Daggers (†) indicate substantial delay in germination and/or growth.
[c] Relative survival from complete to none: +, +–, –+, –.

crenata (50 nuts), *C. mollisima* × *C. dentata* hybrid 'Clapper' (300 nuts), *C. mollisima* (*C. crenata* × *C. dentata*) (three selections, 460 nuts), *C. mollisima* hybrid 'Eaton' (124 nuts), and *C. sativa*-*C. dentata* (? *C. crenata*) (150 nuts) representing a total of 1,696 nuts. In addition, 269 American chestnut nuts collected from a blight-free area (Minnesota) and 75 imported nuts of *C. sativa* purchased from a local store were incubated and examined. None of these 2,040 nuts appeared to be infected with *E. parasitica*.

The effect of different storage temperatures on the development of infections caused by *E. parasitica* was tested using nuts from five trees. Infection was not apparent at harvest, but after 11 wk at 21 C, 12% of 450 nuts apparently were infected (a range of 3–32% among the five trees). No pycnidia were evident on nuts stored at 4 and 13 C for the same time period. However, after additional storage for 7 wk at 4 C and then 4 wk of incubation, 7.7 and 4.8% of these latter nuts, respectively, showed infection.

The effect on infection of heat treating the nuts in water at 50 C for 30 min is presented in Table 2. Nuts from nine trees were treated 1 wk after harvest. The nuts were stored at 4 C until incubated 19 January, and then were examined 4 wk later. Only two of the heat-treated nuts (0.4%) produced pycnidia compared to 142 of the untreated nuts (21.6%). For the untreated nuts, there was a great variation among trees in the percentage of infected nuts (range 1–69%).

The effects of heat treatments for 20–30 min on viability of mycelia and conidia of two normal laboratory cultures of *E. parasitica* are presented in Table 3. Mycelial growth and conidial germination were affected by exposure to 50 C or higher for 30 min. Mycelium was generally killed at 53 C or higher (one sample survived at 55 C), but some spores escaped being killed even at temperatures as high as 60 C for 30 min.

DISCUSSION AND CONCLUSIONS

Our original observation of infected nuts did not preclude the possibility that infection occurred on the ground after release from the burr. Collins (2) had concluded that infection occurred before nut fall. Our evidence also strongly suggests that infection occurs while the nuts are on the tree and in the burr: nuts were harvested in burrs and not allowed to contact the soil; storage and germination media were of Canadian origin, and thus from an area where *E. parasitica* is not present; American chestnuts harvested in a blight-free area (Minnesota), but stored and germinated under the same conditions, were not infected; most nuts were germinated in flats which did not preclude cross contamination within flats, but nuts germinated in separate containers also became infected; infection was greatly diminished in nuts heat treated at harvest; and two nuts were observed to have pycnidia at the time of harvest.

Some nuts infected by *E. parasitica* also contained weevil larvae (*Curculio* spp.), but nuts without weevils were also infected. Therefore, oviposition did not seem responsible for the *E. parasitica* infections. Infection by germination of spores of *E. parasitica* on the style and subsequent growth into the shell is a possibility. Several styles and stigmas from the American trees at Lockwood Farm were surface sterilized and plated onto PDA, but no cultures of *E. parasitica* were isolated.

Infections on nut shells were rarely apparent at harvest, but developed when the nuts were kept warm (~22 C) and moist. Prior cold storage of the nuts was not necessary. Nuts are not normally stored under warm conditions and, thus, infections have not been observed.

The majority of the American chestnut trees in a high-density planting with a high incidence of chestnut blight produced a portion of nuts with *E. parasitica* in their shells. The lack of infections caused by *E. parasitica* in nuts of Chinese, Japanese, and several hybrid chestnut trees growing in the same area indicates that nuts of trees with a measure of blight resistance are resistant to infection.

The means by which hypovirulent strains of *E. parasitica* are maintained and spread in the natural environment is uncertain (8). The lack of dsRNA among the 14 isolates of *E. parasitica* tested for its presence suggests that hypovirulent strains do not play a major role with blight-infected chestnut fruits.

There was no evidence that the presence of chestnut blight in nut shells has any short term deleterious effect on seedlings. However, nuts apparently free from *E. parasitica* at harvest may have latent shell infections in which the pathogen would not be killed by a surface sterilant. Hot water treatments used for killing chestnut weevils (50 C for 30 min) diminish seedborne infections, but cannot be counted on to eradicate *E. parasitica*. These results suggest that the fruit of American chestnut trees, grown where blight is prevalent, have the potential of being infected with *E. parasitica* and of producing fruiting bodies. Importation of such fruit into blight-free areas where blight-susceptible trees are grown, such as the western United States, Argentina, Australia, and New Zealand, poses a risk of introducing the chestnut blight fungus.

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