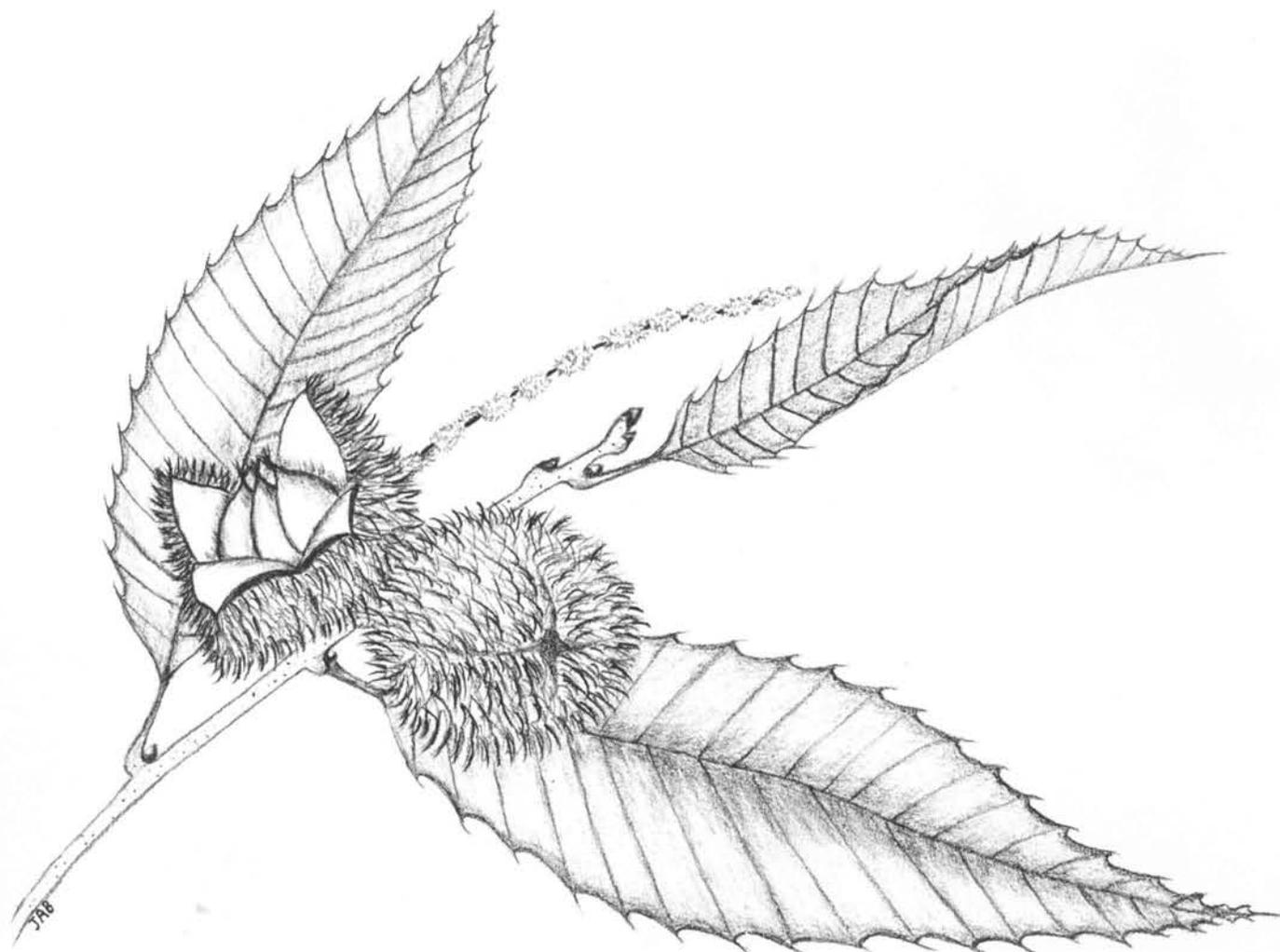


The American Chestnut: New Hope for a Fallen Giant

By Sandra L. Anagnostakis



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I. Background

The American chestnut (*Castanea dentata*) was once the most important hardwood species in the Eastern United States. Its wood was valued for its beauty, and was used extensively for furniture and woodwork. The tall, straight timbers were in great demand for telegraph and fence poles and for railroad ties because chestnut wood resists decay. The bark was an important source of tannin, which was the basis of a large industry. The nuts were food for wildlife, livestock, and people. It is no wonder that many people mourned the passing of this giant.

The blight fungus (*Endothia parasitica*), which was responsible for the loss, seems to have entered the country with Asian chestnut trees brought into New York City around the turn of the century. Although Chinese and Japanese chestnut trees (*C. mollissima* and *C. crenata*) are usually not seriously affected by it, their American cousin is highly susceptible. The canker disease was first reported by Murrill on American chestnut trees in the Bronx Zoological Park in 1906. It spread quickly, and by 1917 most of the trees in Connecticut were dead or dying.

The fungus attacked through wounds: broken branches, breaks in the bark, woodpecker or bark borer holes, etc. Growing out in a circle from the point of infection, the mycelium penetrated between the bark and the wood until it had completely encircled the tree and the tree was effectively "girdled." The tree's efforts

at fighting back included growth of callus (a kind of scar tissue), but the fungus usually penetrated it with ease. Trees on good sites, with adequate light, water, and nutrients resisted the pathogen better than those on poor sites, but even these succumbed eventually.

When the seriousness of the disease became evident, much money and effort went into a campaign to save the chestnut. The Pennsylvania legislature appropriated over \$500,000 to its Chestnut Blight Commission during 1911-1914. Studies on the life history of the fungus continued (Anderson, 1913). Control measures were chiefly restrictions on movement of nursery stock and infected wood into non-infected areas, and clearcutting of chestnut trees ahead of the spreading disease. By 1914 the early optimism of the Pennsylvania Blight Commission had vanished, and the program was declared a failure (Schock, 1914).

Within 40 years the blight fungus had decimated every major stand of American chestnut in the eastern United States. However, the stumps of those trees still produce sprouts from the root collar. This gives us hope that, if a control could be found, the trees might reestablish themselves in the forest.

In Connecticut, a chestnut breeding program was begun in 1931 by A. H. Graves, then employed by the Brooklyn Botanical Garden. He crossed American chestnut trees with Japanese and Chinese trees and hoped the offspring would have the blight resistance of



Figure 1. With the tree passed the classic chestnut cabin, shingled roof, and fence.

the oriental species as well as the form of the American chestnut trees. Graves planted his hybrids on land that he owned in Hamden, CT. This Sleeping Giant Chestnut Plantation came under the management of The Connecticut Agricultural Experiment Station in 1947, and was eventually deeded to the state. Tree

breeding is a long term project, and Graves' work has been continued at the Experiment Station by R. A. Jaynes. Progress has been made, but we are still a long way from producing true breeding forest trees. (Nienstaedt and Graves, 1955; Jaynes and Graves, 1963; Jaynes, 1972, 1978).

Europeans had watched with trepidation the rapid demise of the American chestnut. Thus, their alarm was understandable in 1938 when blight was reported in Northern Italy. Since European chestnut trees (*Castanea sativa*) are as susceptible to *Endothia parasitica* as American trees, an epidemic much like that which had swept this country occurred.

Then, something strange happened. Antonio Biraghi, an Italian plant pathologist, found trees that seemed unusually healthy after repeated attack by the blight fungus (Biraghi, 1951). He found cankers that were healing and that the fungus was restricted to the outer layer of bark on these trees. When Biraghi reported this, few people believed him. However, he persistently claimed a spread of the cure (Biraghi, 1953, 1966, and 1968) and his work attracted the attention and the imagination of a French mycologist, J. Grente. Grente visited Italy in the late 1950's and took bark from healing trees to his laboratory in Clermont-Ferrand. From these he isolated forms of the blight fungus that had reduced virulence. He called these hypovirulent. These hypovirulent forms cured existing blight when they were inoculated into cankers (Grente, 1965). Later, with Berthelay-Sauret, Grente published several reports

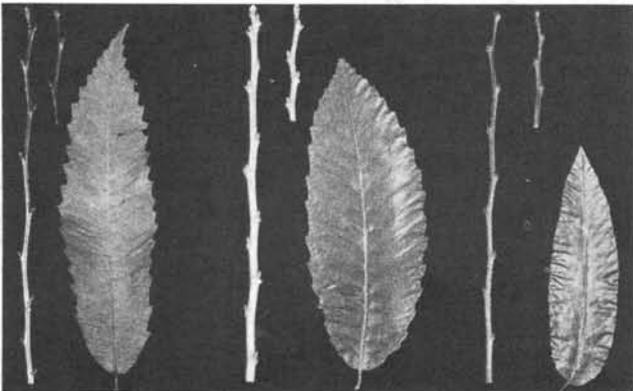


Figure 2. Twigs and leaves of three species of chestnut. Left, the American chestnut leaf has an angular base as compared with the two Oriental chestnuts and the leaf margin is more dentate. The size of the leaf varies and is not critical in identification. Center, the Chinese chestnut twigs have a light, yellowish-buff winter twig color; there are simple hairs at the tip of the twig and the leaf is broad. Right, the Japanese chestnut has rounded buds and a leaf that is narrow and bristle-tipped with a crenate margin (from Jaynes, 1978).

on these unique strains (Grente, 1971; Grente and Sauret, 1969a,b; Berthelay-Sauret, 1973). Once a canker had been successfully cured by treatment with a hypovirulent (H) strain, much of its fungal mycelium seemed to be converted to the H form. Grente and Berthelay-Sauret described the behavior of their strains in culture: H-strains segregated, yielding normal looking strains; but normal, virulent strains (V) never segregated to yield H cultures. They suggested that, in the host, hyphae of the V strain anastomosed (fused) with hyphae of the introduced H strain and some genetic determinant in the cytoplasm was transferred that converted the V strain to H as it moved through the mycelium. Grente's recent work suggests that determinants can be transferred most often between strains from the same geographic area, and that transfers rarely occur between strains from different areas (Grente, 1975; Grente and Berthelay-Sauret, 1978a,b).

Richard Jaynes at The Connecticut Agricultural Experiment Station had read Biraghi's papers and was prompted to look into the phenomenon by Grente's 1965 paper. Grente sent a French V strain and two H strains, which the Station imported under a permit from the USDA Plant Quarantine Division. We grew seedling American chestnut trees in the greenhouse and inoculated them with French and American V strains of *E. parasitica*, with French H strains, and with pairs of V and H strains. We found (Anagnostakis and Jaynes, 1973) that the French strains behaved as described by Grente (Grente, 1965; Grente and Sauret, 1969a,b). Results of two pairings of an American V strain with a French H strain were less dramatic; one of the trees died, while the other showed extensive

fungal growth, but did not wilt, even after 100 days had passed. The tree wound was heavily calloused, and we made isolations of *E. parasitica* before the trees were autoclaved to satisfy plant quarantine requirements. The reisolated strain looked like the original French H strain when grown on agar media in the laboratory and, as was reported for the original (Grente and Sauret, 1969a,b), single conidia spread on agar media yielded a variety of colony forms.

As our results looked promising, we obtained permission (1973) to conduct experiments on field-grown trees at our Experiment Station farm. N. Van Alfen and R.A. Jaynes made many paired inoculations of American V strains with the reisolated H strain and obtained better disease control than we had seen initially. Tests with strains identifiable by nuclear genes (Puhalla and Anagnostakis, 1971) proved that hypovirulence is determined by genes in the cytoplasm of *Endothia parasitica* and is transferred by hyphal anastomoses. Double-stranded ribose nucleic acid (dsRNA) is present in the cytoplasm of H strains but not in the cytoplasm of V strains. dsRNA is the genetic material of most fungal viruses.

The Station obtained quarantine permission to conduct more extensive experiments after this work was published (Van Alfen *et al*, 1975). R.A. Jaynes tested 42 kinds of native and exotic woody plants for susceptibility to disease caused by V or H strains of *Endothia parasitica*. These included plants from 17 families. The only ones showing growth of the fungus were American chestnut (*C. dentata*), "Crane" Chinese chestnut (*C. mollissima*), "Eaton" chestnut (*C. mollissima* hybrid), and a Connecticut Japanese-American-Chinese hybrid chestnut (Jaynes,



Figure 3. An old stump of American chestnut (not visible under the leaves) has sprouted from the roots. The blighted sprout at the right shows the typical orange pustules of the blight fungus on the shrunken bark. The sprout stem on the left is heavily calloused.

Anagnostakis, and Van Alfen, 1976). Thus, we were assured that we could test these fungi in wooded areas without harming other species. Our work now could diversify to the real world of sprout clumps of American chestnut trees in forests, to more work on the growth and behaviour of our V and H strains on synthetic media in the laboratory, and to more biochemical tests for dsRNA and a search for the presence of virus-like-particles in our *Endothia* cultures.

We can cure a given canker on a tree, and are making progress in understanding the nature of hypovirulence (Anagnostakis, 1978a). We now know that:

- Hypovirulence is a disease or group of diseases of the fungus *E. parasitica* that reduces its pathogenicity but not its vigor as a saprophyte.
- It is controlled by genetic determinants in the cytoplasm of the fungus.
- The determinants are probably on, or associated with, dsRNA.
- All hypovirulent strains examined contain dsRNA (Day, *et al*, 1977).
- The dsRNA is associated with clubshaped virus-like-particles in at least one strain (Dodds, 1977, 1978; Day and Dodds, in press).

Most of this knowledge has come from the study of H strains from France and Italy and American H strains

that we have derived from them. We wondered why this cure had arisen in Europe and not here. Then, in 1976, a woman in Michigan who had read an article from the *New York Times* about our work, was cross-country skiing on a golf course when she saw in a small forest-island blighted chestnut trees that looked as if they were healing. The trees were hardly beautiful, but they were surviving massive infection. She sent us leaves that proved that the trees were American chestnuts, and pieces of bark from the gnarled and twisted trunks. Our first native American hypovirulent strains were isolated from those samples. Later Elliston and Dodds found that these H strains differ from the European H strains, but they too contain dsRNA and can cure existing blight infections (Elliston and Dodds, 1978). Recently trees in another part of Michigan, in Pennsylvania, and in Virginia have yielded similar strains.

There is great variation in appearance and pathogenicity among our H strains. John Elliston (1978) has found pathogenicity ranging from zero up to normal among 20 strains that contain dsRNA. Most of these produce asexual spores when they grow in chestnut trees, but a few produce sexual spores. Since early workers in America concluded that sexual spores were the primary source of new infections and spread of the blight, we wonder how the H strains in Italy have spread so quickly and efficiently.



Figure 4. Chestnut producing areas of Italy before (left) and after (right) the advent of chestnut blight. Blight was first observed in Busalla (B) and spread rapidly. Biraghi first observed cankers healing in Masone, not far from Busalla.

II. Current Research

We introduce H strains into cankers by removing 4-6 plugs of bark around the circumference of the canker and then filling the hole with mycelium of one or more H strains in agar. The holes are covered with waterproof brown paper tape to prevent desiccation (Puhalla and Anagnostakis, 1971). Our first major test in a forest involved 300 trees on state and private land and one H strain—an American strain derived from the original French strain. As Jaynes and Elliston have reported (1978), 86% of the cankers were controlled in the first year. However, only 13% of the treated cankers remained controlled after 3 years. New cankers had formed at other points on the “cured” trees and the H strain did not spread from tree to tree or even on the same tree. This proved that hypovirulence could control natural infections on American chestnut sprouts. However, long term survival of the trees and natural spread of H strains had not been achieved. Other evidence (Elliston and Jaynes 1977) suggested the presence of a system of vegetative incompatibility in the fungus which could prevent anastomoses between strains. This led to new studies in the laboratory.

Andes (1961) described interactions between single-ascospore (sexual spore) clones of normal virulent *E. parasitica* on potato dextrose agar. Mycelia of his pairs

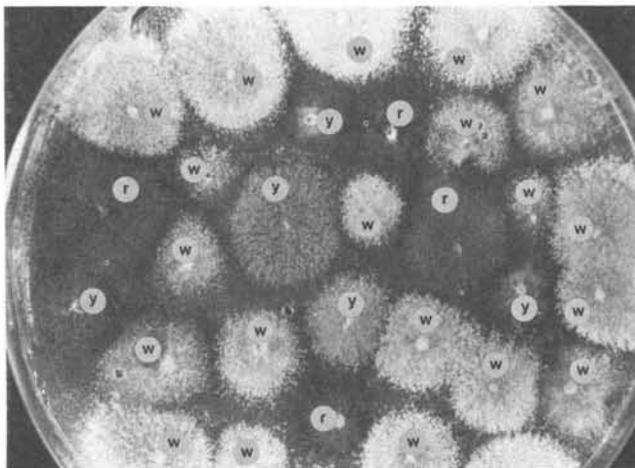


Figure 5. Colonies of *Endothia parasitica* from single conidia of a white French hypovirulent strain. White and red-orange hypovirulent strains and normal (yellow-orange) strains grown on Difco PDA in the light.



Figure 6. Blight canker on an American chestnut. The canker was inoculated with a hypovirulent strain of the blight fungus at the points marked X. One year later, when the photograph was taken, the canker remains the same size.

of clones had merged, formed a zone of inhibition, or formed a ridge of asexual fruiting structures between them. Since hyphal anastomosis is required for transfer of the determinants for hypovirulence (Van Alfen *et al*, 1975), it appeared that vegetative (mycelial) incompatibility might explain the occasional failure of cure with H strains.

If incompatible V strains and normal strains isolated from H Strains are paired on potato dextrose agar medium (Difco Co.) a line, or barrage zone, of inhibition will form between them. In some cases, ridges of asexual fruiting bodies (pycnidia) form along these lines. Strains within a given vegetative compatibility (v-c) group simply merge with each other

Table 1. Chestnut blight canker control (+) in American chestnut stems. Hypovirulent strains of *Endothia parasitica* in three vegetative compatibility (v-c) groups were paired with virulent strains.

v-c group	Virulent Strains		Hypovirulent Strains		
	stock number		EP 4b7 v-c 10	EP 43 v-c 5	EP 14 v-c 8
1	EP 106		-		
2	EP 107 or 41		-	+	-
3	EP 108		+		
4	EP 109		-		
5	EP 110 or 42		-	+	-
6	EP 111		+	-	+
7	EP 114		+ ^w		
8	EP 6		-		
9	EP 59 or 39		-	-	+
10	EP 67		+ ^w	?	?
11	EP 46		+ ^w	-	+ ^w
12	EP 62		+ ^w		
14	EP 89		+ ^w	+ ^w	+
16	EP 29		+ ^w		
17	EP 78		+		
18	EP 74		-		
19	EP 37		+	+ ^w	+
20	EP 15		-		
21	EP 76		+		
22	EP 58		+ ^w	+ ^w	+ ^w
23	EP 5		-	-	+
24	EP 30		+ ^w	-	+
25	EP 38		-	+ ^w	-

^w means weak control

on the agar and the hyphae anastomose (Anagnostakis, 1977, 1978b). So far, we have found 50 v-c groups (36 in Connecticut). If we are to cure chestnut blight in the forest, we must understand this incompatibility.

We now have many H strains from France, Italy, and North America with very different phenotypes (J. E. Elliston, 1978) that represent several v-c groups. Three of these have been paired with V strains in 23 different groups in American chestnut stems. Cankers were formed by some pairs but not by others (see Table 1). Grente and Sauret (1969a), reported that only 6 of 50 (12%) pairs of V and H strains from the same region formed cankers in *C. sativa*. If the H and V strains came from different regions, 124 of 170 (73%) of the pairs formed cankers. They noted that anastomoses between an H strain from Italy and a V strain from France resulted in the degeneration of cytoplasm. They proposed that this was a manifestation of an incompatibility, which might explain their results in the field. Grente (1975; Grente and Berthelay-Sauret, 1978a,b) found that repeated pairings between incompatible V and H strains can sometimes produce H strains with the compatibility type of the V parent. We have isolated such H strains from incompatible pairings in the host that resulted in cure (see Table 1).

Caten (1972) suggests that "vegetative incompatibility [in fungi] will markedly reduce the spread of suppressive, cytoplasmic genetic elements, including viruses, from strain to strain in nature," and that it can be viewed as a cellular defense mechanism. Partial protection may be occurring in *E. parasitica*, with differences at a few (or certain) gene loci allowing



Figure 7. A large American chestnut tree being checked for hypovirulent strains of the blight fungus. Madison, Virginia, 1978.



Figure 8. *Endothia parasitica* strains on agar medium. Mycelia of strains in the same vegetative compatibility groups merge together, barrage lines form between those which are different.

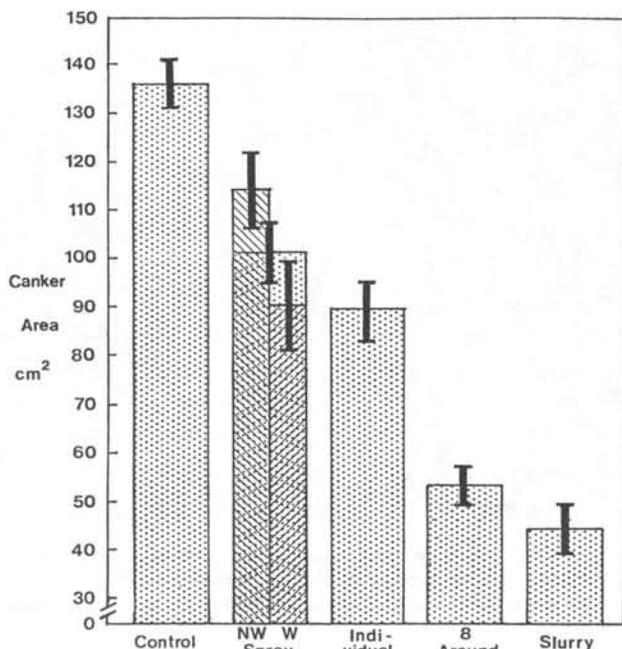


Figure 9. 1977 Field test: Arrest of virulent cankers on American chestnut sprouts treated with eight hypovirulent strains. Standard error of the mean indicated. Control = cankers received no H treatment; NW = cankers sprayed with conidia but not wounded; W = cankers wounded and sprayed with conidia; Individual = each canker inoculated with a single H; eight around = all eight H inoculated individually around the canker; Slurry = mix of all eight H inoculated in four places around the canker (from Jaynes & Elliston, 1978).

anastomoses which do not lead to cell death (e.g. Caten, 1973; Handley and Caten, 1975). It is also possible that nuclear genes, which suppress incompatibility, may be present in some strains, or that the determinants for hypovirulence may be suppressive. Nuclear genes that eliminate vegetative incompatibility have been reported in other fungi (e.g. in *Neurospora* by Newmeyer, 1970, and in *Podospora* by Esser, 1968).

In the summer of 1977 we planned field tests that included H strains from several v-c groups and with different abilities to grow and sporulate in the host. Fifteen forest plots were chosen, each with 24 sprout clumps of American chestnut. Uniform infections with normal *E. parasitica* were induced, using bark pieces

from a natural infection in each area. The treatments were begun 5 weeks later. A group of eight H strains, which included French-derived American, native American, and Italian, was chosen. These represented six v-c groups and varied in pathogenicity and ability to sporulate. Four treatment methods were used:

1. Spores from eight H strains mixed in water sprayed on the cankers
2. Four plugs of an H strain put into holes around each canker
3. One plug of each H strain put into eight holes around each canker
4. A mixture of all eight H strains (a mycelial slurry with agar) put into four holes around each canker.

That fall, we found that all treatments had limited the size of the cankers as compared to the untreated controls. The mixture (4) was the most effective, reducing canker areas from an average of 135 cm² to 45 cm² (Jaynes and Elliston, 1978). The cankers treated were caused by *E. parasitica* strains in 25 compatibility groups; five were the same as v-c types among the H strains used in the experiment. In the test plots where only one H strain was used per canker it was possible to see which v-c groups were controlled by which H strains (Table 2). Canker areas showed a wide range among these treatments. Cankers in v-c group 43 were not controlled well by any of the single H strains (nor, incidentally by the mixture, where the average final canker area was 70 cm²). Cankers in v-c group 24 were controlled by H strain EP 14 (in v-c 8), but not by two other H strains. We plan to test mixtures of different H strains to find the best method of curing the largest number of cankers.

However, one major problem remains. In Italy, we are told, blight is no longer a problem due to the natural spread of hypovirulence (Bonifacio and Turchetti 1973; Turchetti, 1978; Mittempergher, 1978). We have seen no evidence of such natural spread of biological control in our New England forest test plots. It is possible that some vector, such as bird or an insect, may be responsible for the rapid spread of the curing strains in Italy, and these vectors may not be present here (Day, 1978). In addition, H cultures have never been found to produce the airborne sexual spores (ascospores) that are most likely the source of new infections. This suggests

Table 2. Average areas (cm²) of *Endothia parasitica* cankers in the 1977 field test of Jaynes and Elliston. These data are from the part of the test in which hypovirulent strains were used one strain per canker.

Vegetative compatibility of cankers treated	Hypovirulent strains and their vegetative compatibility groups						
	EP 9 v-c 20	EP 14 v-c 8	EP 50 v-c 10	EP 49 v-c 12	EP 61 v-c 12	EP 60 v-c 9	EP 90 v-c 9
v-c 5					42		20
v-c 17					28		60
v-c 19			71	155	152	114	
v-c 24	90	22		189			
v-c 35	177	98		91			
v-c 43	62	64	57	127	122	53	
v-c 46				91			67

that the dsRNA or virus-like-particles are excluded by sexual reproduction in *Endothia*. We will be watching our 1977 forest plots for the next several years for evidence of spread.

We are hoping that our team effort will lead to rapid progress in finding the best way to control chestnut blight in the United States and bring this magnificent tree back to importance in our forests.

Acknowledgements

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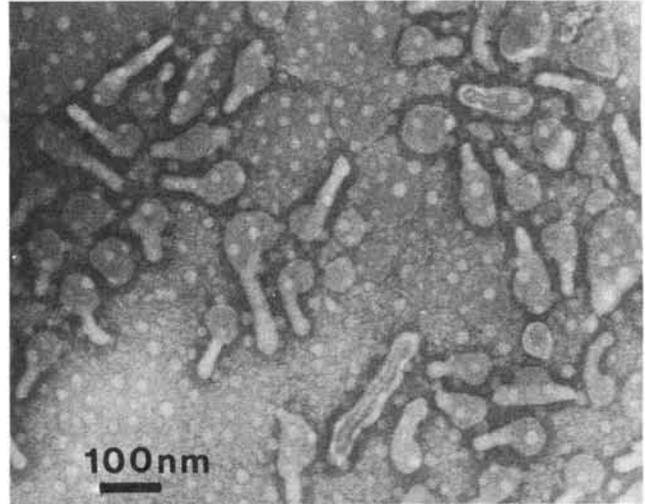


Figure 10. Electron micrograph of club-shaped virus-like particles purified from a French hypovirulent strain of *E. parasitica* (strain 3) negatively stained in 2% phosphotungstic acid (pH 7.0).

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