lar energy loss. Since a continuum of intensities occurs in the spectrum underneath the characteristic absorption edges (see Fig. 7), two images must be taken to obtain an elemental map: one with a narrow band of energies just above the specific absorption edge, and one just below this edge in energy. The difference between the images gives the map or spatial disrtibution of the chosen element. Figure 8 shows the net elemental distributions for silicon, oxygen, and carbon in a section of cells from a bean plant at a lesion produced by an incompatible rust infection (*21*). While silicon could be, and was, analyzed by x-ray microanalysis, the distribution of oxygen and of carbon (or the lack of the latter in this case), as well as the spatial resolution evident in the images, can only be obtained by the imaging of scattered electrons.

At higher resolutions, we have analyzed the phosphorus distributions in individual leaflets of embedded and sectioned phospholipid-containing membranes (*26, 28*), as well as the phosphorus distribution in DNA in nucleosomes (*29*). Preliminary results (Fig. 9) indicate that the distribution of phosphorus in the ribosome, delineating perhaps the ribosomal RNA, may also be amenable to study (*21*).

The good spatial resolution obtained, 0.3 to 0.5 nm (*28*); the high detection sensitivity measured, 50 atoms of 2×10^{-21} gram of phosphorus; and the short exposure time necessary, typically 5 to 15 seconds (*26*), make this technique one to two orders of magnitude better than x-ray microanalysis on each of these points. Moreover, if present applications by individual users of the device at the level of the cell membrane, the nucleosome, the Golgi apparatus, microvilli, fungal infection, bone formation, and others are an indication, the uses of energy analysis of scattered electrons in biology will be far-reaching.

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Biological Control of Chestnut Blight

Sandra L. Anagnostakis

Chestnut blight, a classic among plant diseases, is caused by an introduced fungus that has nearly eliminated its host: the American chestnut tree (*1*). Since chestnut blight was first detected in the United States at the turn of the century, there has been no evidence of the development of genetic resistance to the disease and chemical control methods have not proved useful.

After the first infected trees were found in the Bronx Zoo in 1904 (*2*), the lethal canker organism *Endothia parasitica* (Murr.) And. spread in ever-widening circles to encompass all of the natural range of the tree. By 1950 an estimated 9 million acres (about 3.6 million hectares) of American chestnut trees were dead or dying (*3*).

American chestnut [*Castanea dentata* (Marsh) Bork.] was once the most important hardwood species in the eastern United States. Its beautiful wood was used extensively for furniture and woodwork. The tall, straight, decay-resistant timbers were in great demand for telegraph and fence poles and for railroad ties. The tanin extracted from chestnut bark and wood was the basis of a large leather tanning industry. The nuts were food for wildlife, livestock, and people. It is no wonder that many people mourned the passing of this giant.

The fungus attacks through wounds: broken branches, breaks in the bark, or woodpecker or bark borer holes. Growing out from the point of infection, the mycelium grows in the bark and outer sapwood until it has completely encircled and effectively "girdled" (Fig. 1) the tree or branch. The trees may resist by production of callus tissue, but the

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fungus usually penetrates the callus with ease (*4*). Trees on good sites, with adequate light, water, and nutrients may resist the pathogen better than those on poor sites, but even those on good sites succumb eventually.

When the seriousness of disease became evident, much money and effort went into a campaign to save the chestnut. The Pennsylvania legislature appropriated over $275,000 for its Chestnut Blight Commission from 1911 to 1913 (*5*). Studies on the life history of the fungus continued (*6, 7*). Control measures were chiefly restrictions on movement of nursery stock and infected wood into noninfected areas and the clear-cutting of chestnut trees ahead of the spreading disease. By 1914 the early optimism of the Pennsylvania Chestnut Blight Commission had vanished, and the program was declared a failure (*8*).

Summary. After 77 years of being attacked by the chestnut blight fungus, American chestnut trees continue to sprout from gradually declining root systems. The blight fungus in Italy is now associated with virus-like agents that limit its pathogenicity, and attempts have been made to introduce these controlling agents into the blight fungus in the United States. If a way can be found to help the spread here of strains of the fungus with controlling agents, it may be possible to save the American chestnut trees in our eastern forests.

However, the roots of killed trees produce sprouts (*9*). Thus, if means of control can 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 Botanic Garden. He crossed American chestnut trees with Japanese and Chinese trees, hoping the offspring would have the blight resistance of the Oriental species as well as the form of the American chestnut trees. Graves planted his hybrids on land that he owned in Hamden, Connecticut. 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 Richard A. Jaynes. Progress has been made, but we are still a long way from producing true breeding forest trees (*10*).

Many fungicidal and fungistatic chemicals have been applied to blight cankers over the years but none has been useful for long-term therapeutic treatment of the disease. Jaynes and Van Alfen (*11*) found that annual pressure injections of methyl-2-benzimidazole carbamate (MBC) solutions would maintain field-grown American chestnut trees for 3 to 4 years, but foliage injury also resulted. Delen (*12*), in Turkey, made tests of several related benzimidazole compounds in vitro and in the greenhouse with European chestnut trees (*Castanea sativa*, Mill.) which are also very susceptible to chestnut blight. Although drenching the soil around the roots provided some control of artificially induced cankers, tests in vitro showed that *E. parasitica* can acquire persistent resistance to the benzimidazoles which he tested.

Europeans watched with trepidation the rapid demise of the American chestnut. Their alarm was understandable in 1938 when blight was reported in northern Italy. An epidemic occurred much like that which had swept this country (*13*). Antonio Biraghi, an Italian plant pathologist, followed the progress of the disease. The first blight cankers were found in the area around Genoa, in the northern province of Udine, and in a small area in Avellino (see darkly shaded areas in Fig. 2). By 1950 Biraghi found blight widely distributed in the northern and southern chestnut-growing regions (lightly shaded areas in Fig. 2). He recommended cutting the dead trees off at the ground to reduce the level of inoculum (*14*). Then, in 1951 Biraghi noticed a chestnut coppice near Genoa that seemed unusually healthy. In his 1953 paper (*15*) he said that this coppice "was once severly damaged by *Endothia parasitica* and . . . it was impossible then to find any living shoot older than four or five years . . . [in 1951] about 85 percent of the shoots were infected by *Endothia parasitica*, but only a few showed the usual symptoms characteristic of blight. . . . " He found cankers that were healing and noticed that the fungus was restricted to the outer layer of bark on these trees. His persistent claims attracted the attention of a French mycologist, J. Grente. Grente visited Italy in the early 1960's and took bark from healing trees to his laboratory in Clermont-Ferrand. From these samples he isolated forms of the blight fungus that looked different and that had reduced virulence. He called these hypovirulent (*16*). These hypovirulent forms cured existing blight when they were inoculated into cankers.

Later, with Sauret, Grente published several reports on these unique strains (*17–21*). Once a canker had been successfully cured by treatment with a hypovirulent (H) strain, much of the fungal mycelium from the original infection seemed to be converted to the H form. Grente and 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 of the H strain was transferred that converted the V strain to H.

Richard Jaynes at the Connecticut Agricultural Experiment Station had read Biraghi's reports and was prompted to examine the phenomenon described in Grente's 1969 papers. Grente sent a

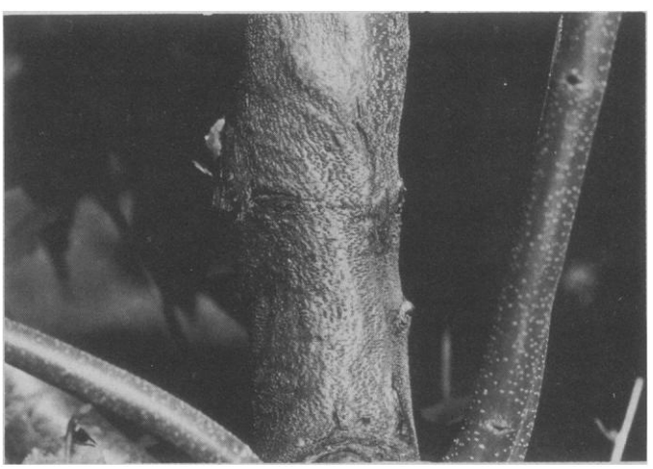

Fig. 1. A typical chestnut blight canker. The fungus *Endothia parasitica* on American chestnut, *Castanea dentata*. Ridges of callus can be seen in progressive rings out from the central (left in picture) site of infection. This defense by the tree is easily penetrated by the fungus, which grows as a tight fan of mycelium under the bark. Orange pustules of mycelial stroma break through the bark and the asexual spores (conidia) are formed in pycnidia. Sexual spores (ascospores) form in perithecia in the same tissue late in the growing season.

Fig. 2. Spread of chestnut blight in Italy as observed by Professor Antonio Biraghi in 1938 and late 1949. [Redrawn from Biraghi (*14*)]

French V strain and two H strains, which the station imported under a permit from the U.S. Department of Agriculture 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 that the French strains behaved as described by Grente. Results of two pairings of an American V strain with a French H strain were less dramatic. One of the trees died; the other showed extensive fungal growth, but did not wilt, even after 100 days had passed (*22*). 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 strain, the uninucleate conidia produced a variety of colony forms when they were spread out and germinated on agar media.

Field Experiments with European H Strains

Because our results looked promising, we obtained permission (1973) to conduct experiments on field-grown trees at our experiment station farm. Van Alfen and Jaynes made many paired inoculations of American V strains with the reisolated H strain and obtained quicker disease control than we had seen initially. Tests with strains identifiable by nuclear genes proved that hypovirulence is cytoplasmically determined in *E. parasitica* and is transferred by hyphal anastomoses. Peter R. Day (*23*) found that double-stranded ribonucleic acid (dsRNA) was 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 permission from the USDA Plant Quarantine Division to conduct more extensive experiments after this work was published (*23*). Jaynes tested 42 kinds of native and exotic woody plants for susceptibility to disease caused by V or H strains of *E. 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-grown Japanese-American-Chinese hybrid chestnut (*24*). Thus, we were confident 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 behavior of our V and H strains on synthetic media in the laboratory, and to more tests for dsRNA and a search for the presence of virus-like particles in our *Endothia* cultures.

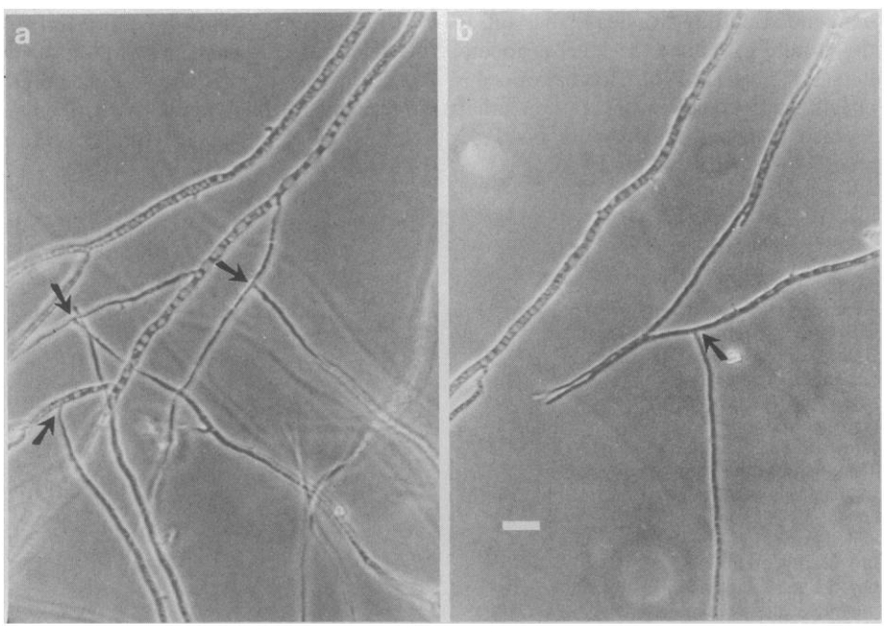

Fig. 3. Two examples of anastomoses between hyphae of vegetatively incompatible strains of *E. parasitica* on 4 percent water agar at 25°C in the dark. Note the shrunken appearance of the cells at the arrows. Scale bar, 50 micrometers.

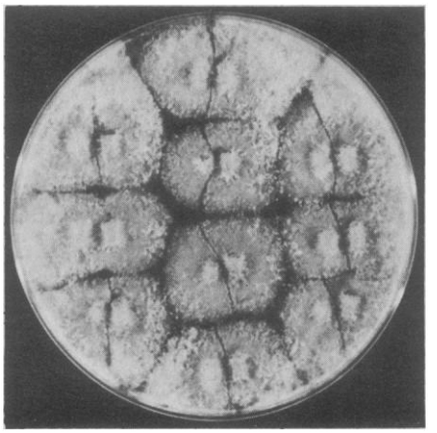

Fig. 4. A test of vegetative compatibility (v-c) with *E. parasitica*. An unknown strain (left member of each pair) paired with the standard testers of v-c groups 1 to 10. The medium is potato dextrose agar (Difco) with methionine (100 milligrams per liter) and biotin (1 milligram per liter), and the plate was incubated at 28°C in the dark. The unknown strain has merged with the v-c 2 tester (second down on left) and so is in v-c group 2. It has formed a barrage line with the nine other testers.

We now know that:

1) Hypovirulence is a disease or group of diseases of the fungus *E. parasitica* that reduces its ability to kill susceptible chestnut tree hosts (*23*).

2) It is controlled by genetic determinants in the cytoplasm of the fungus (*23, 25*).

3) The determinants are probably on, or associated with, dsRNA (*25, 26*).

4) All hypovirulent strains examined contain dsRNA (*25*).

Thus for the first time since the advent of chestnut blight in this country we have something to use for therapeutic treatment of blight cankers.

We introduce H strains into cankers by removing four to six plugs of bark, each 10 millimeters in diameter, around

the circumference of the canker with a cork borer, then filling the holes with mycelium of one or more H strains in agar. After they are filled, the holes are covered wtih waterproof brown paper tape to prevent desiccation. Our first major test in a forest involved 300 trees on state and private land and an American H strain derived from the original French strain. As reported by Jaynes and Elliston (*27*), 86 percent of the cankers were controlled in the first year. However, new cankers that formed at other points on the "cured" trees were not cured and were lethal. This proved that hypovirulence could control natural infections on American chestnut sprouts, but long-term survival of the trees and natural spread of H strains had not been achieved.

Our failure to control all of the treated cankers with a single H strain was reminiscent of the report by Grente and Berthelay-Sauret (*17*) that only 6 of 50 pairs (12 percent) of V and H strains from the same region formed cankers in *C. sativa*, whereas 124 of 170 pairs (73 percent) from different regions formed cankers. They also noted that anastomoses formed on agar media in the laboratory between an H strain from Italy and a V strain from France resulted in the degeneration of cytoplasm (Fig. 3). They proposed that this was a manifestation of an incompatibility. Since hyphal anastomoses are required for transfer of the determinants for hypovirulence, we also thought that a genetic system of vegetative isolation in the fungus might explain the failures of cure with H strains.

If vegetatively incompatible V strains are paired on potato dextrose agar medium (Difco) in the dark, a line, or barrage zone, of inhibition will form between them (*28*). In some cases, ridges of asexual fruiting bodies (pycnidia) form along these lines (Fig. 4). Strains within a given vegetative-compatibility (v-c) group simply merge with each other on the agar and the hyphae anastomose. So far, we have found 77 v-c groups, 67 of them in Connecticut (*29*).

From our laboratory studies we now estimate that at least seven nuclear genes determine these v-c groups (*30*). The genes we have examined in detail all behave heterogenically; that is, strains are vegetatively compatible only if they have the same alleles at a given gene locus. Parental lines with the same alleles at all v-c genes yield progeny that are all in the same v-c group. If progeny fall into two v-c groups, the parents had different alleles at a single locus. Difference at two loci would yield four progeny types, three would yield eight, and so on. By making crosses of this kind and examining the progeny for v-c type we have been able to assign tentative genotypes to some of our v-c types, and we can begin to talk about failure of H transmission in terms of degree of gene difference. Grente found that repeated laboratory pairings between incompatible V and H strains sometimes produced 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. Peter Day and I used Grente's method of pairing V and H strains on sterile cellophane placed on the surface of agar medium, and found that we could convert V strains to H morphology even if they were vegetatively incompatible (*26*). Not all incompatible combinations resulted in conversion, but about 20 to 50 percent of the time we were successful (Fig. 5).

Caten (*31*) 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 anastomoses which do not lead to cell death. It is also possible that nuclear genes, which suppress vegetative incompatibility, may be present in some strains or that the determinants for hypovirulence may be suppressive.

This led to a field test of pairs of strains different from each other at zero, one, two, or at least five v-c gene loci. Four randomized replicates with H determinants from two sources gave us data which clearly show the effect of this vegetative isolation system (*29*). Several V strains were inoculated first, then the incipient cankers were treated 2 weeks later with V strains or H strains. The treatment with V strains had little effect and the treatment with H strains was effective in varying degrees depending on the number of v-c genes different between the strains.

Comparison of European and American H Strains

Most of our work has been done with H strains from France and Italy and American H strains that we have derived from them. We wondered why hypovirulence had appeared in Europe and not in the United States. Then, in 1976, we heard from a woman in Michigan who had read a newspaper article about our work. She had been 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. We found that these H strains differ from the European H strains, but that the American H strains also contain dsRNA and can cure existing blight infections (*25*). Recently, trees in another part of Michigan as well as in Pennsylvania, Tennessee, and Virginia have yielded similar strains (*32, 33*).

There is great variation in appearance

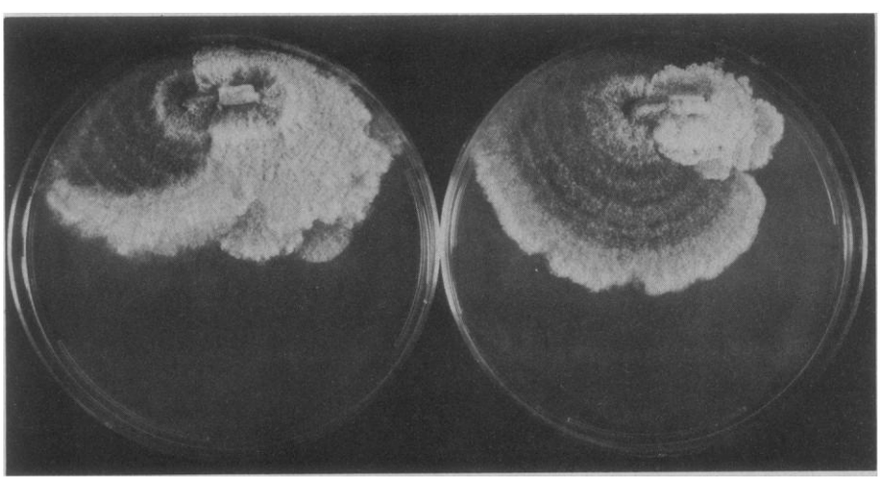

Fig. 5. When white European H strains are paired with normal strains on sterile cellophane over agar medium (16 hours of light per day, 28°C) the normal strains may be converted to H morphology. In these pairs the strains on the left are normal. The normal in the left plate shows conversion as a wedge of white mycelium beginning at about the second-day ring of growth. In the plate on the right there has been no conversion (the mycelial edge usually lacks pigment).

and pathogenicity among our H strains (Fig. 6). Elliston has found pathogenicity ranging from zero up to normal among 20 strains that contain dsRNA (*34*). Most of these produce asexual spores when they grow in chestnut trees, but few produce sexual spores. The European slow-growing H strains with no (or little) pigment (white) produce conidia which grow into (i) normal, (ii) white H, (iii) deeply pigmented H, or (iv) intermediate white types. This segregation among strains growing from uninucleate conidia suggests cytoplasmic control of the morphology. Second-generation conidia from the normal types yield only normals, and conidia from the white or intermediate types can yield all four types again. The hardest to understand are the deeply pigmented H strains which Grente called *jaune régénéré* (JR). Conidia from JR strains always yield only JR types, never normals or others (*35*, *36*). When white H strains are paired with normal V strains, the converted V mycelia are white, fast, or slow-growing. When JR strains are paired with V strains, the converts are always white, never JR (*26*, *35*).

The dsRNA of these European H types has been characterized by Dodds (*32*) as type I dsRNA (from our original white strain from Grente) and type II dsRNA (from all other H strains from Europe: white, JR, and intermediate). Dodds has found pleomorphic membrane-bound structures in both white and JR European H strains (*37*). These structures contain the dsRNA of these strains but seem to have no protein associated with them (*38*). They do not resemble, therefore, any of the typical virus-like particles which have been found in fungi so far, but are like structures found in some diseased mushrooms by Lesemann and Koenig (*39*). The structures found in diseased mushrooms have not been tested for dsRNA.

The American H strains present a very different picture. Elliston (*40*) found that a highly debilitated strain from Michigan segregated four types of mycelia when conidia were plated: normal types, intermediate types, and two kinds of highly debilitated types. Conidia from normals yielded only normals. Conidia from the intermediates yielded normals and intermediates. Conidia from one kind of highly debilitated strain yielded only normals and highly debilitated types, whereas conidia from the other (indistinguishable from the first) yielded normals, intermediates, and highly debilitated types. Ellison believes that two kinds of H determinants are present in the original strain (Fig. 6). Segregation of morphological types from conidia of other American H strains is being studied. Dodds (*32*) found the dsRNA in American H strains from Michigan and Virginia to be very different from the European H strains in the banding patterns obtained by polyacrylamide gel electrophoresis. He calls this type III dsRNA. He was unable to isolate pleomorphic membrane-bound structures from American H strains (*41*).

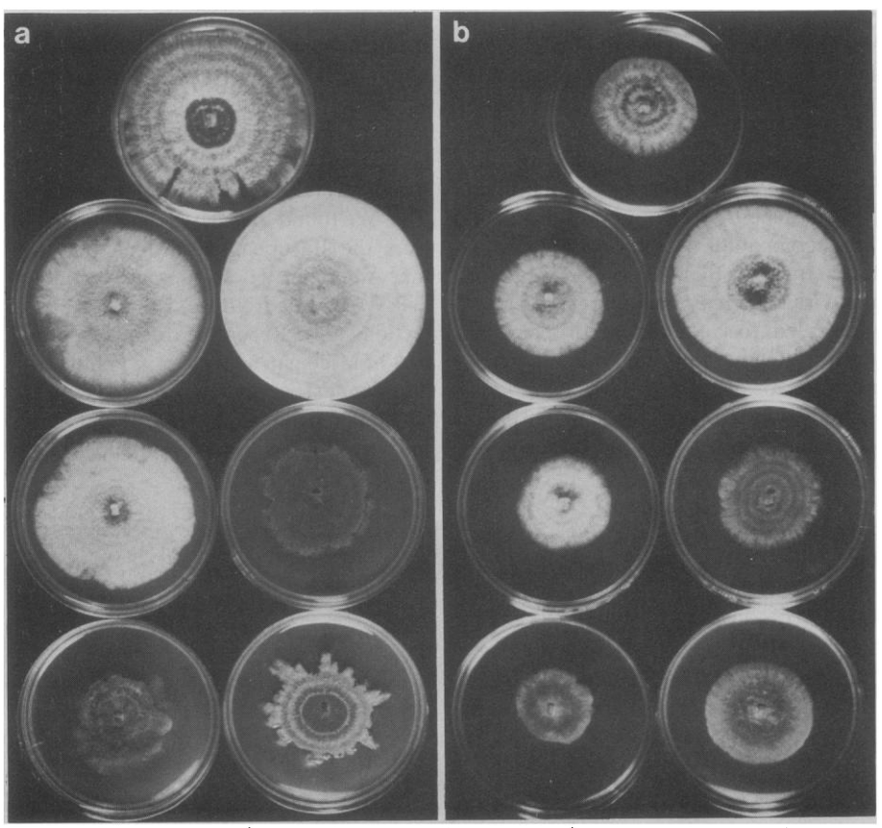

Fig. 6. Morphology of virulent and hypovirulent strains of *E. parasitica* under two sets of growth conditions. The strains are as follows: Top, EP67 (ATCC No. 38753) normal, virulent. Second row, left, EP67 with Italian H agents, "slow white," and right, EP67 with Italian H agents, "fast white." Third row, left, EP67 with French H agents, and right, EP420 a *jaune régénéré* type of H strain from Italy. Bottom row, left, EP67 with one of the Michigan H agents, and right, EP67 with the second Michigan H agent. The cultures were grown for 7 days on Difco potato dextrose agar under the following conditions: (a) 100 milligrams of methionine and 1 milligram of biotin per liter, 30 milliliters of medium per plate, with the plates left unsealed and incubated at 28°C with 16 hours of white fluorescent light per day; (b) 100 milligrams of methionine and 10 micrograms of biotin per liter, 20 milliliters of medium per plate, with the plates sealed with Parafilm and incubated at 20°C with 16 hours of white fluorescent light per day.

In order to find the most efficient way to use H strains therapeutically on cankers caused by *E. parasitica* strains in diverse v-c groups, 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 virulent *E. parasitica* were induced by using bark pieces from a natural infection in each area. The treatments were begun 5 weeks later. A group of eight H strains was chosen. These included French-derived American, native American, and Italian, they represented six v-c groups, and varied in pathogenicity and ability to sporulate. Four treatments methods were used:

1) Spores from eight H strains mixed in water and 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.

We found that all treatments limited the size of the cankers compared to the untreated controls (*27*). The mixture (treatment 4) was the most effective, reducing canker areas from an average of 135 square centimeters to 45 square centimeters. The cankers treated were caused by *E. parasitica* strains in 25 v-c 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 possi-

ble to see which v-c groups were controlled by which H strains. Canker areas showed a wide range among these treatments. We plan to test mixtures of different H strains to find the best method of curing the largest number of cankers.

Present Efforts to Control Chestnut Blight

Grente now uses mixtures of up to ten different H strains of *E. parasitica* grown on a paste of cornstarch and packaged in aluminum tubes like toothpaste (*42*). If a French plantation owner reports that blight has reached his farm, field agents are dispatched to treat the cankers by making holes in the bark around the cankers and applying the H mixture. Grente says that they get good control in these commercial plantations if ten trees per hectare are treated for each of 3 years, then five trees per hectare for 2 to 3 years (*42, 43*). He is now trying to find reasons for the apparent spread of the "cure" in the French orchards (*42*).

In Italy there is a renewed interest in the chestnut orchards which produced the much sought after marron types of chestnut. Since the trees producing this type of nut are all hybrids, they can only be propagated by grafting them onto wild stocks. For this grafting process, blight is still a serious problem and Turchetti (*44*) has found that even H strains can infect newly made graft wounds and kill scions. Some method must be found to protect grafts made by plant propagators in chestnut nurseries and by farmers in the woods next to their orchards.

However, the dense Italian forest stands of *C. sativa* are being used again as a timber source because the blight cankers rarely kill them (*45*). Although we can now treat blight cankers on *C. dentata* individually and successfully cure them, we would like to see the spread and establishment of these H agents in our American forests.

We have found no evidence of such natural spread of the controlling agents in our New England forest test plots. There are many differences between our situation and that in France and Italy. The trees are a different species. Chestnut forests in Italy are nearly monocultures, as are the French orchards, whereas we have mixed stands of hardwoods. Although some areas in Virginia have dense stands of American chestnut sprouts, comparable to the density in Italy, the fungus must generally move farther here to get from host to host. It is also possible that some carrier, such as a bird, mammal, or insect, may be responsible for the spread of the H strains in France and Italy, but may not be present here. All of these questions must be faced in our future research.

We plan to try repeated therapeutic use of mixtures of H strains in our forest test plots, in dense plantings, and in orchard plantings of young American chestnut trees. Meanwhile we will work in the laboratory to understand the phenomenon of cytoplasmically determined hypovirulence in *E. parasitica*.

To summarize, the biological control of chestnut blight has occurred naturally in Italy, is used with some success in France, but has not yet been sustained in the United States. To succeed in the United States, we must find or produce stable H strains of *E. parasitica* that (i) can survive in nature without killing *C. dentata*, (ii) are present in broad ranges of vegetative compatibility, and (iii) are conducive to natural spread.

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