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CHESTNUT BLIGHT: THE CLASSICAL PROBLEM OF AN INTRODUCED PATHOGEN[1]

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Populations of plant pathogenic fungi which evolve with their hosts are often relatively harmless to the populations of plants that they infect, allowing reproduction and survival of both. The same pathogens may have a serious impact on a newly introduced host species or on native species where the pathogen is newly introduced. These plant pathological disasters have led to the establishment of plant quarantine regulations in most countries, but some of our early mistakes are still with us. The chestnut tree blight fungus, first found in the United States in early 1900, is a good example of this. *Cryphonectria parasitica* (Murr.) Barr is a classical plant pathogen, known to many scientists and lay people for its disastrous effects on the American chestnut tree population, its rapid epidemic spread after it was introduced into the United States, and its persistence in its new habitat.

THE TREES

American chestnut trees [*Castanea dentata* (Marsh.) Borkh.] were once a major component of the hardwood forests of the eastern United States (Gravatt, **1949**). In the southern part of its range the tree grew to 37 meters tall and 1.5 meters diam (Buttrick, **1925**), and could increase in diameter at the rate of 2.5 cm per year on good sites (Buttrick, **1925**; Kuhlman, **1978**). The trees formed sprouts from the root collar after cutting, thus replacing themselves after harvest (Graves, **1926**).

The wood seasoned well and was extremely resistant to decay due to tannins found in both bark and wood (Cook and Wilson, **1915**; Fowler, **1944**; Nienstaedt, **1953**). It was used for construction, woodwork, furniture, fencing, boxes, barrel staves, railroad ties, telegraph poles, mine timbers, and musical instruments (Gibson, **1913**; Clapper and Gravatt, **1943**; Saucier, **1973**; Kuhlman, **1978**; Rice *et al.*, **1980**). The tannins were extracted from the bark and wood and formed the basis of a large leather tanning industry (Fowler, **1944**; Saucier, **1973**).

Chestnut burrs have extremely sharp spines, which distinguish them easily from horsechestnut burrs. Nuts (three per burr) are normally released at the first fall frost. These were an important source of food for wildlife, domestic livestock, and humans. They were also used by country people as barter for other supplies (Rice *et al.*, **1980**).

The stature of the trees made them useful in urban plantings for shade (Emerson and Weed, **1918**), and it was on shade trees that the fatal chestnut blight disease was first seen in the United States. American chestnut trees lining the avenues of the New York Zoological Garden were reported to be dying in 1905 by H. W. Merkel (**1905**).

The symptoms were bark cankers, wilting of the distal foliage, and the formation of epicormic sprouts directly below the canker (FIG. 1) (Murrill, **1906a**; Metcalf, **1912**; Anderson, **1914**). Orange fungal stromata pushed through the epidermis and in these, pycnidia and perithecia were found. In moist conditions sticky orange tendrils of conidia were extruded from the pycnidia (Rankin, **1914**; Shear *et al.*, **1917**). Chestnut blight cankers can be most easily seen on the bark of juvenile trees when the bark is wet. At that time the orange color of the epidermis, where mycelium has penetrated, is usually obvious. Cankers are not easily seen on mature bark, where often the first sign of blight infection is the formation of the epicormic shoots below the canker. These form because *C. dentata* is ring-porous, with larger vessels formed in the xylem in the spring than during the rest of the year. Most of the vessels from one year are occluded by the next year. If the cambium is unable to make new vessels because it was killed by chestnut blight or some other injury, the tree forms shoots to bypass the blockage.

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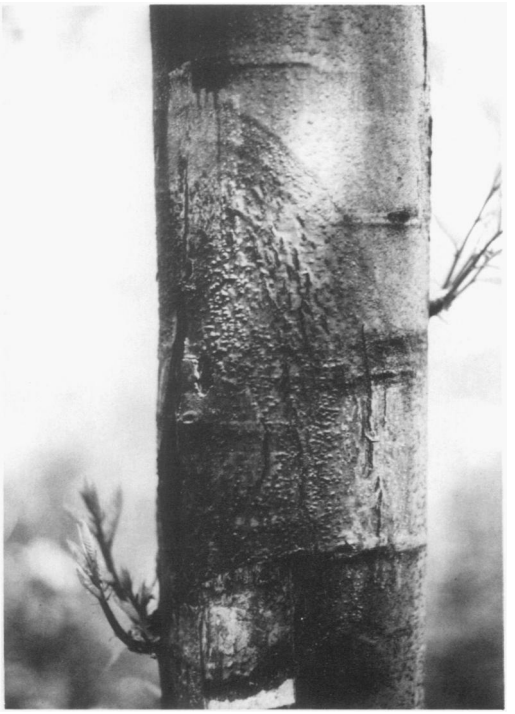

FIG. 1. Chestnut blight canker caused by *Cryphonectria parasitica* (Murr.) Barr on *Castanea dentata* (Marsh.) Borkh. Note that stromata have erupted through the lenticels and the tree has formed epicormic shoots. Discoloration of the xylem by phenolic compounds can be seen where the bark is peeled away.

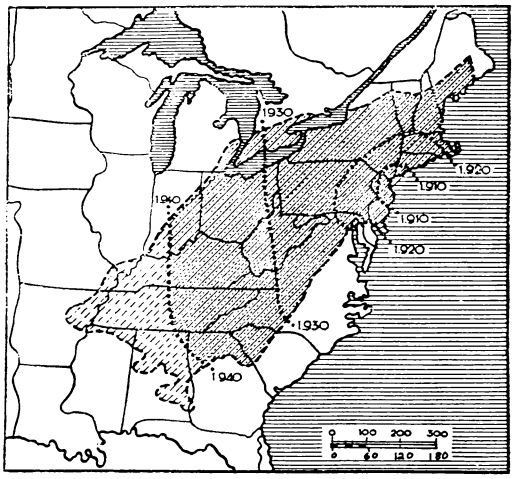

FIG. 2. Map with the native range of *Castanea dentata* outlined. The dotted lines with dates show the progress of the epidemic of *Cryphonectria parasitica* through the population. The top numbers on the scale are miles, those on the bottom kilometers (from Gravatt, 1949).

Pruning and a spray program using Bordeaux mixture failed to control or contain the disease (Murrill, **1906b**). After *C. parasitica* moved out of New York State an epidemic resulted which could not be stopped. It progressed rapidly through the entire native range of *C. dentata,* from Maine to Alabama, and west to the southern edge of Michigan (FIG. 2). Its spread proceeded at the rate of about 37 kilometers per year, and within 50 years about 3.6 million hectares of American chestnut trees were dead or dying (Anonymous, **1954**). The value of the standing chestnut timber in Pennsylvania, North Carolina, and West Virginia alone was estimated at about $82.5 million in 1912 (Detwiler, **1912**; Giddings, **1912**; Buttrick, **1925**). A series of reports was issued to inform the public and many suggestions were made for local control (Metcalf, **1907**; Metcalf and Collins, **1909**, **1911**; Mickleborough, **1909**; Pennsylvania Chestnut Tree Blight Commission, **1912a**, **b**; Giddings, **1912**; Rogers and Gravatt, **1915**; Gravatt and Marshall, **1926**; Baxter and Gill, **1931**). Suggestions were also made by state foresters about the possible use of the dead timber, and the length of time it could be expected to remain sound and marketable (New Hampshire Forestry Commission, **1914**).

European chestnut trees, *Castanea sativa* Mill., are not really natives of Europe. They were found by the Romans growing around the Black Sea and planted wherever the Romans colonized (FIG. 3) to provide a very important food and timber supply. European chestnut trees are very similar to American chestnut trees, and when blight finally was found in Italy in 1938, the ensuing epidemic was much like that in the United States (Woodruff, **1946**; Pavari, **1949**). 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. By 1950, Biraghi found blight widely distributed in the northern and southern chestnut-growing regions. He recommended cutting the dead trees off at the ground to reduce the level of inoculum (Biraghi, **1950**). Blight may have invaded Spain at about the same time but positive identification was not reported. It reached France about 1946, Switzerland (Tessin) about

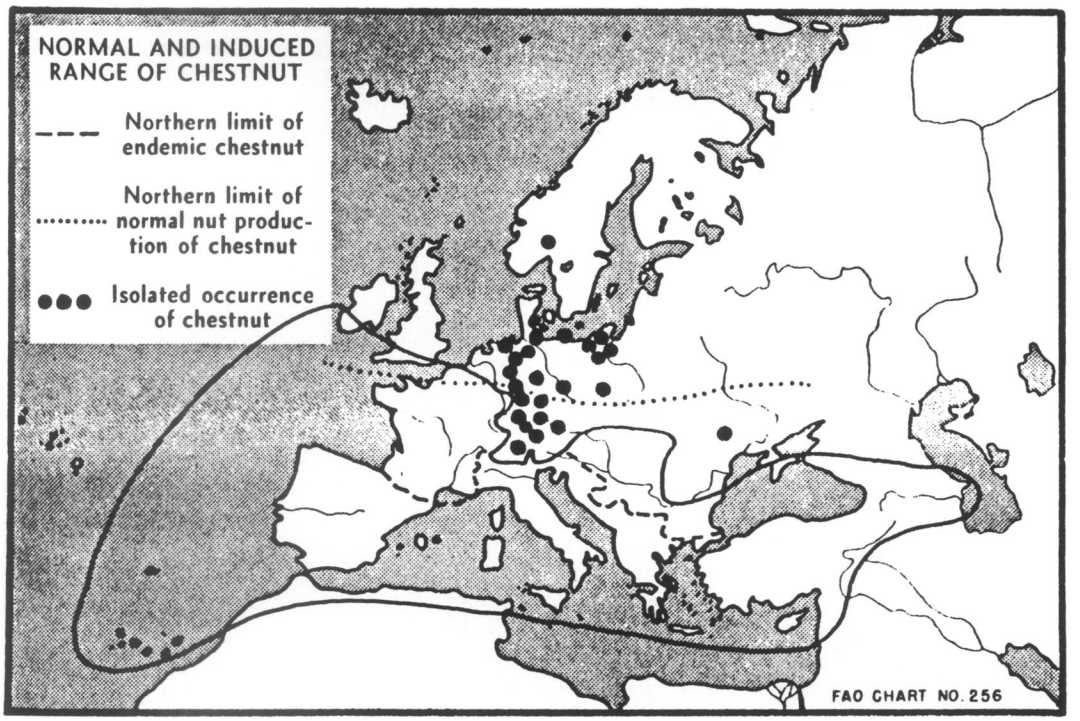

FIG. 3. Map showing native and introduced range of *Castanea sativa* in Europe. Reproduced from FAO chart 256.

1951, Greece by 1964, and Turkey by 1967 (Biraghi, **1950**, **1951**; Bazzigher, **1957a**; Kailides, **1967**; Delen, **1975**; Grente, **1981**; Turchetti, **1978**). Shain has compared the rate of spread in Italy and the United States (Shain, **1982**). *Castanea sativa* is a little more resistant to blight than *C. dentata* (Berry, **1960**; Hebard, **1982**). This lengthens the time from infection to death, and may have an effect on inoculum potential (sporulating stromata present for a longer time). Hebard (**1982**) has considered the implications of this for epidemiology.

Attempts to produce American chestnut trees resistant to the fungus have been made since the early work of Clapper and Gravatt (**1943**). These have included hybridizations with the more resistant Asian species (Clapper and Gravatt, **1943**; Graves, **1950**; Jaynes, **1964**, **1972**; Jaynes and Dierauf, **1982**), irradiation of American nuts (MacDonald *et al.*, **1962**; Dietz, **1978**) and breeding of the remaining large American trees (Thor, **1973**). Since tree breeding and selection is a long-term project, it may be years before trees with good form and suitable resistance are developed by these methods. The work of A. H. Graves was continued by R. A. Jaynes until 1983. C. R. Burnham in Minnesota proposes to continue this work, and is preparing a review on this subject for *Plant Breeding Reviews*.

Early breeding and selection of European chestnut trees resulted in distinct timber and nut tree types. Since all were susceptible to blight, G. Bazzigher started breeding trees for blight resistance in 1951, and today has about 40,000 registered selections including trees with medium to high blight resistance (Bazzigher, **1981**).

THE FUNGUS

Taxonomists discussed the causal fungus and its origins. Murrill (**1906b**) described the fungus as a new species, *Diaportha parasitica* Murrill. Anderson and Anderson (**1912a**, **b**) then transferred it to the genus *Endothia*. A series of laboratory studies produced descriptions of its growth characteristics *in vitro*, and compared it to similar fungi (Anderson and Anderson, **1912a**, **b**, **1913**; Shear and Stevens, **1913**; Anderson, **1914**; Shear *et al.*, **1917**). More recent taxonomy

has included a comparison of related species from the southern U.S. (Sawyer, **1975**) and a very thorough study in Japan (Kobayashi and Ito, **1956**) lists potential hosts, as does Nash (**1983**). Roane and Stipes evaluated the genus *Endothia* in 1978 and decided that the genus name was correct (Roane and Stipes, **1978a**). However, when Barr reevaluated the Diaporthales she renamed the genus *Cryphonectria* (Barr, **1979**). In a monograph recently published by the American Phytopathological Society Roane and Griffin have defended the use of the older name (Griffin *et al.*, **1986**). Micales feels that Barr is correct (Micales, **1985**; Micales and Stipes, **1987**).

Early mycologists and plant pathologists thought the origin of the chestnut blight fungus must have been in China and Japan because of the resistance to the disease shown by Chinese and Japanese chestnut trees (Metcalf, **1908**; Clapper, **1952**). When requests for information directed to scientists in those countries proved fruitless, the well known plant explorer David Fairchild was consulted (Fairchild, **1939**). Explorer Frank Meyer who worked for Fairchild had sent chestnut trees from China to the United States (Meyer, **1911**). In 1913, he was in eastern China and Metcalf, Shear, and Fairchild sent him a bark sample as an example of the disease and asked him to look for similar lesions on Chinese chestnut trees (Fairchild, **1913**). He sent two samples of blight from Chinese chestnut to Fairchild, and Shear, Stevens, and Metcalf isolated the fungus from the samples and inoculated it into American chestnut trees near Washington, D.C. (a second introduction of the pathogen which is seldom noted) (Shear and Stevens, **1913**; Fairchild, **1939**). The cultural characteristics and pathology proved these isolates to be the same fungus as that causing the blight disease on American chestnut trees. Meyer later found the disease in Japan and sent samples from there to the United States as well (Shear and Stevens, **1916**).

The mechanism of dissemination was initially thought to be wind dispersal of conidia (Murrill, **1906b**). Mickleborough (**1909**) discussed both conidia and ascospore formation. H. R. Fulton is reported to have conducted experiments on spore movement and concluded that conidia in tendrils would not be carried far by wind (Heald *et al.*, **1915**). The conidia remain viable in the soil for a long time and might be dispersed by wind-borne dust (Heald and Gardner, **1914**). Rankin called attention to the forcible expulsion of the ascospores from perithecia and suggested that they might be carried long distances by the wind (Rankin, **1912**, **1913**). The work of others later confirmed this feature of ascospore expulsion (Anderson, **1914**; Shear *et al.*, **1917**). The best documented work on wind dissemination was that of Heald *et al.* (**1915**) who showed that slow drying of bark after a rain allowed ascospore discharge to continue for up to 14 hours. After a dry period, as little as 4.5 mm of rain was sufficient to cause copious expulsion of ascospores. Their data suggest that conidia are not prevalent in the air at any time.

Although the conidia may not be wind-borne, they have been recovered in large numbers from the surfaces of insects, birds, and mammals. The first work on insects as carriers of the chestnut blight fungus was that of F. C. Craighead (**1912**). An abstract by Studhalter (**1914**) reports that conidia were more frequently recovered from the surface of insects than ascospores. The final report of the Pennsylvania Chestnut Tree Blight Commission discusses insects (Ruggles, **1914**), and a more complete report was issued the next year (Studhalter and Ruggles, **1915**). Occasional additions to our knowledge of this subject were made over the following 70 years (Rand and Pierce, **1920**; Anagnostakis, **1982a**; Wendt *et al.*, **1983**; Russin *et al.*, **1984**). The most important insect carriers are probably the wound-makers *Strophiona nitens* Forster [formerly *Anoplodera* (*Leptura*) *nitens*] and *Ectoedema phleophaga* Bsk. (the chestnut bast-miner), and the grazer *Leptostylus maculata* Say.

Birds and mammals also pick up *C. parasitica* as they move over cankers with erumpent stromata. In 1914, birds frequenting cankers were shot and washed, and the wash-water examined for propagules of *C. parasitica.* Heald and Studhalter (**1914**) found that 19 of the 36 birds carried the fungus. A similar technique was used for birds and small mammals by Scharf and DePalma (**1981**), who found that two of 64 birds and two of 89 animals carried *C. parasitica.*

Laboratory cultures of *C. parasitica* have been described as having mycelia that were at first white and then yellow (Anderson, **1914**; Shear *et al.*, **1917**). The pigments have been well studied and appear from yellow in acid solution, to magenta in basic solution (Anderson, **1914**; Roane and Stipes, **1978b**).

Light has been reported to induce asexual sporulation in *C. parasitica* (Anderson, **1914**). When

the mycelium is grown in an alternating light/dark regime pycnidia form on the part of the mycelium young enough to be induced by the light. This results in concentric rings of pycnidia separated by sterile mycelium of hyphae which were too old for pycnidial induction when the light period began. In continuous light, pycnidia are fewer and scattered. In continuous darkness the pycnidia appear much later, even fewer are formed, and they are scattered over the mycelium. Pycnidia also form in large numbers along the edge of a knife-cut through mycelium, and along barrage lines between vegetatively incompatible strains (see below).

The fungus responds to temperature in a similar manner whether growing *in vivo* or *in vitro*. *Cryphonectria parasitica* will grow at an optimum rate over a broad range of temperatures, and light appears to have no effect on this rate. The rate of canker expansion in American chestnut trees is approximately $\frac{1}{10}$ that of the rate of colony expansion on potato dextrose agar at the same temperature. The fungus appears to have no temperature "memory"; that is, change in ambient temperature elicits an immediate response in the form of changed growth rate (Anagnostakis and Aylor, **1984**).

The physiology of the fungus growing *in vitro* was examined by Bazzigher who reported his findings in a series of papers (Bazzigher, **1953**, **1955**, **1957a**, **b**, **c**, **1958**) that provided a sound basis for future experiments. Media for laboratory growth have been described (Puhalla and Anagnostakis, **1971**; Anagnostakis, **1982c**), and the genetics of *C. parasitica* recently reviewed (Anagnostakis, **1984b**, **c**).

HYPOVIRULENCE

With the history of chestnut blight in the U.S. as an example, and with the obvious similarity of the epidemic in Italy before them, plant pathologists pessimistically assumed that the European chestnut trees were doomed to be reduced to short-lived understory shrubs like their American counterparts. However, only 15 years after the epidemic in Italy had begun, Biraghi reported that Italian trees were healing themselves and recovering (Biraghi, **1951**). In his 1953 paper he said that a coppice near Genoa "was once severely damaged by *E. 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 *E. parasitica,* but only a few showed the usual symptoms characteristic of blight . . ." (Biraghi, **1953**). He found cankers that were healing and noticed that the fungus was restricted to the outer layer of bark on these trees. French mycologist J. Grente visited Italy in 1964 with a group of FAO foresters 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 (Grente, **1965**). These hypovirulent forms cured existing blight when they were inoculated into cankers.

Later, with Sauret, Grente published several reports on these unique strains (Grente and Sauret, **1969a**, **b**; Grente, **1971**, **1975**; Berthelay-Sauret, **1973**). Once a canker had been successfully cured by treatment with a hypovirulent (H) strain, much of the fungal mycelium in 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 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.

In 1971 we wrote to Grente and he sent us a French V strain and two H strains, which The Connecticut Agricultural Experiment Station imported under a permit from the U.S. Department of Agriculture Plant Quarantine Division. We made test inoculations on seedling American chestnut trees in the greenhouse and found that the French H strains could also limit expansion of cankers caused by American V strains on American trees (Anagnostakis and Jaynes, **1973**). We made isolations of *C. parasitica* before the trees were autoclaved to satisfy plant quarantine requirements. The reisolated American 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.

Because our results looked promising, we obtained permission (in 1973) to conduct experiments on field grown trees at our Experiment Station farm. Van Alfen and Jaynes (Van Alfen

et al., **1975**) 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 *C. parasitica* and is transferred by hyphal anastomoses. Van Alfen *et al.* (**1975**) found that double-stranded ribonucleic acid (dsRNA) was present in the cytoplasm of H strains but not in the cytoplasm of V strains.

The Experiment Station then obtained permission from the U.S. Department of Agriculture Quarantine Division to conduct more extensive experiments. Jaynes (Jaynes *et al.*, **1976**) tested 42 kinds of native and exotic woody plants for susceptibility to disease caused by V or H strains of *C. 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* Bl.), "Eaton" chestnut (*C. mollissima* hybrid), and a Connecticut-grown Japanese-American-Chinese hybrid chestnut developed by Graves. Thus we were reasonably confident that we could test these fungi in wooded areas without harming other species.

Our work then diversified 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 in our cultures. We now know that:

1) Hypovirulence is a disease, or a group of diseases of the fungus *C. parasitica,* that reduces its ability to kill susceptible chestnut tree hosts (Van Alfen *et al.*, **1975**).

2) It is controlled by genetic determinants in the cytoplasm of the fungus (Day *et al.*, **1977**).

3) The determinants are probably on, or associated with, dsRNA (Day *et al.*, **1977**; Anagnostakis and Day, **1979**; Anagnostakis, **1981**; Fulbright, **1984**).

4) All hypovirulent strains examined contain dsRNA of molecular weight higher than that of typical fungal viruses (Day *et al.*, **1977**; Dodds, **1980a**; Fulbright, **1984**).

5) The dsRNA is surrounded by membrane (possibly of host origin) and is not encapsidated (Dodds, **1980b**; Van Alfen, **1982**).

To use H strains for therapeutic treatment of blight cankers we remove plugs of bark around the circumference of the canker with a cork borer, and fill the holes with mycelium of one or more H strains in agar. 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. In this test, 86 percent of the cankers were controlled in the first year (Jaynes and Elliston, **1980**). However, new cankers that formed at other points on the "cured" trees were not cured, and proved lethal. This demonstrated 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.

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, bark samples from American chestnut trees in Michigan yielded our first native American hypovirulent strains of *C. parasitica.* We found that these H strains differ in phenotype from the European H strains, but that the American H strains also contain high molecular weight dsRNA and can cure existing blight infections (Day *et al.*, **1977**). Recently, trees in another part of Michigan as well as in Pennsylvania, Tennessee, and Virginia have yielded similar strains (Jaynes and Elliston, **1982**; Fulbright *et al.*, **1983**; Fulbright, **1984**; Elliston, **1985a**, **b**).

There is great variation in appearance and virulence among our H strains. Elliston has found virulence ranging from zero up to normal among 20 strains that contain dsRNA (Elliston, **1978**). Most of these produce asexual spores when they grow in chestnut trees, but H mycelia do not normally produce perithecia either *in vivo* or *in vitro,* and H determinants have not been found among their progeny. Although H strains can serve as male parents in crosses (the conidia function as spermatia), the H determinants from hypovirulent male parents are not found among the ascospore progeny (Anagnostakis, **1984a**). 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 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) and Bonifacio and Turchetti called "pigmentato" (P) (Grente and Sauret, **1969b**; Bonifacio and Turchetti, **1973**). Conidia from these strains always yield similar types, never normals or others. The morphology segregates in crosses as if controlled by a single nuclear gene. If this is a phenotype resulting from a nuclear gene mutation, its frequency is astonishingly high (Grente, **1981**). It is possible that this genotype represents the integration of dsRNA or DNA copies of dsRNA into the nuclear genome (Anagnostakis, **1984a**).

The H type that has been studied most is that found most often among isolates from healing cankers in Italy. Dodds (**1980a**) refers to the dsRNA from such strains as Type 2 dsRNA, based on banding patterns in polyacrylamide gel electrophoresis. Strains of this kind grow a little more slowly *in vitro* than V strains with the same nuclear genome (Anagnostakis and Aylor, **1984**). They also produce less pigment (*i.e.,* are "white") and fewer pycnidia and conidia than normal strains with the same nuclear genotype. L'Hostis *et al.* (**1985**) found no sequence homology between dsRNA from European and American H strains. Paul and Fulbright (**1987**) found the same thing, but did find homology between dsRNAs from some American strains.

The only biochemically defined characteristic that we have been able to link with the H genome is reduced production of oxalate on laboratory media (Havir and Anagnostakis, **1983**). We found that three virulent strains excreted oxalic acid when grown on a number of substrates, and that the oxalate was produced via the enzyme oxaloacetate acetylhydrolase (E.C.3.7.1.1). Three hypovirulent strains (nuclear genotypes corresponding to the virulent strains) contained a substance which behaved like a heat-stable protein or protein complex which interfered with the activity of this enzyme (Havir and Anagnostakis, **1985**). Oxalate production by other fungi has been linked to their pathogenicity (Noyes and Hancock, **1981**; Maxwell, **1973**). When oxalate production by *C. parasitica* was first reported (McCarroll and Thor, **1978a**) it was proposed that oxalic acid produced in the host might chelate and remove calcium from cell wall and membrane components in host bark, and reduce the ambient pH. This would then allow the polygalacturonase produced by the fungus to function. The action of this and other enzymes could produce the gelatinous zone seen in advance of the mycelium in cankers (McCarroll and Thor, **1978b**, **1985a**, **b**). Further possibilities relating to the role of oxalate production in fungi were discussed by Jennings (**1984**).

VEGETATIVE INCOMPATIBILITY

Jaynes and Elliston (**1980**) found that one could not cure all cankers in a forest plot with a single, or a few, H strains. This was reminiscent of the report by Grente and Sauret that only 6 of 50 pairs (12%) of V and H strains from the same region formed cankers in *C. sativa,* whereas 124 of 170 pairs (73%) 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. They proposed that this was a manifestation of an incompatibility (Grente and Sauret, **1969b**). 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 of *C. parasitica* are paired on potato dextrose agar medium (Difco) in the dark, a line, or barrage zone, will form between them (Andes, **1961**; Anagnostakis, **1977**). In some cases, ridges of pycnidia form along these lines (FIG. 4). Strains with the same vegetative-compatibility (v-c) type form a group whose members simply merge with each other on the agar and the hyphae anastomose. A barrage zone between incompatible cultures has many dead cells where anastomoses have formed and a lethal reaction has resulted. This cell death was first studied in *Neurospora crassa* by Garnjobst and Wilson (**1956**).

From laboratory studies we now estimate that at least five nuclear genes determine these v-c types in *C. parasitica* (Anagnostakis, **1980**, **1982b**). The genes we have examined in detail all behave heterogenically; that is, strains are vegetatively compatible only if they have the same alleles at all given gene loci. 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

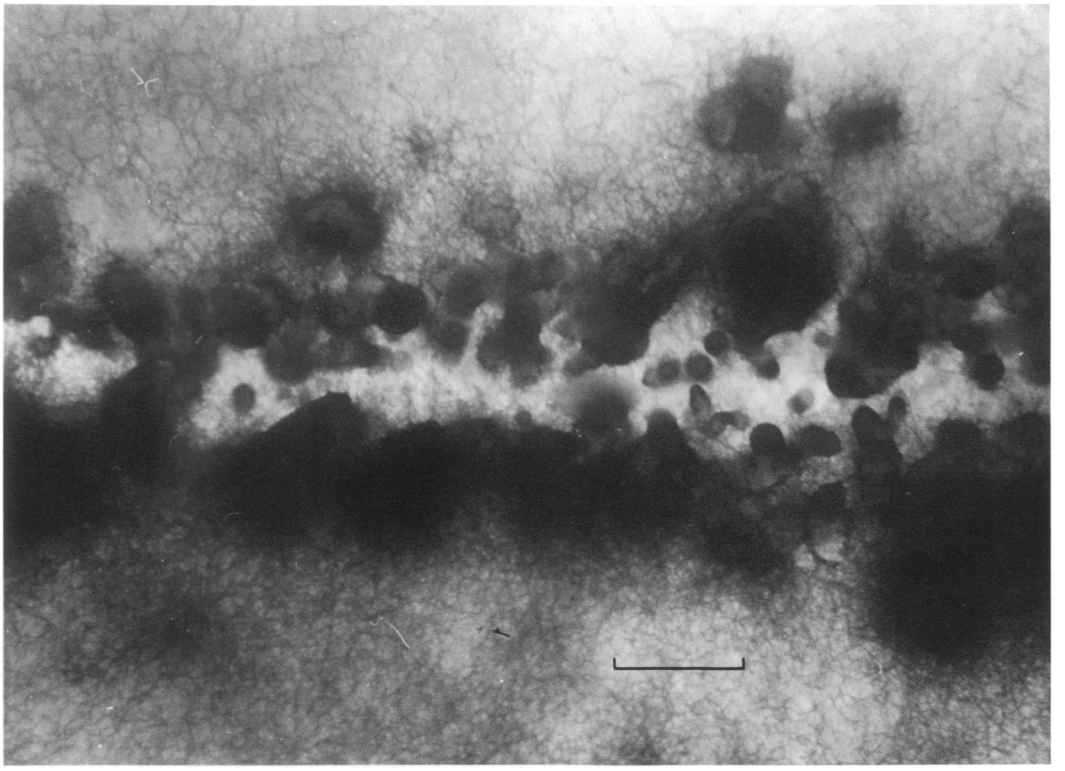

FIG. 4. Close-up view of a barrage with pycnidia between vegetatively incompatible strains of *Cryphonectria parasitica* on agar medium. Bar marker represents 500 microns.

been able to assign tentative genotypes to some of our v-c types (Anagnostakis, **1982b**), and we can begin to talk about failure of H transmission in terms of degree of gene difference. In another Ascomycete, *Podospora anserina* (Ces. in Rabenh.) Niessl, detailed genetic studies have revealed nine gene loci involved in vegetative incompatibility (Esser, **1974**; Labarère *et al.*, **1974**; L. Belcour, pers. comm.). Six of the loci have two alleles each in nature and three loci have five, four, and three alleles each in nature. Making the calculation of how many genotypes are possible, the total number should be 7680.

We know very little about the mechanisms that lead to visible barrage. Some genes may control aversion, while others control speed of cytoplasmic death after anastomoses are formed. Such a system has been described in the Myxomycete *Didymium iridis* (Ditmar) Fries by Ling and Ling (**1974**). In *C. parasitica* incompatible anastomoses must sometimes be viable for some length of time, because we can demonstrate that H determinants are sometimes passed between paired vegetatively incompatible strains. The chance of transfer may depend on how many v-c genes have different alleles in the two strains. Grente found that repeated laboratory pairings between incompatible V and H strains sometimes produced H strains with the compatibility type of the V progenitor, and that such H strains could sometimes be isolated after incompatible pairings in the host resulted in cure. When we used Grente's method of pairing V and H strains on sterile cellophane placed on the surface of agar medium, we also found that we could sometimes convert V strains to H morphology even if they were vegetatively incompatible (Anagnostakis and Day, **1979**). Not all of our incompatible combinations resulted in conversion, but about 20 to 50 percent of the time we were successful. Kuhlman's work on conversion has led to a concept of "hypovirulence conversion compatibility" (Kuhlman and Bhattacharyya, **1984**; Kuhlman *et al.*, **1984**). Strains with different genotypes,

giving them different v-c types, may still be able to pass hypovirulence determinants, and this is the important thing for control and spread in the field. Recent data on the diversity of vegetative compatibility in Connecticut make it clear that biological control probably cannot be established as easily in Connecticut as in Europe where there is little diversity (Anagnostakis *et al.*, **1986**; Anagnostakis and Kranz, **1987**). More data are needed on hypovirulence conversion compatibility to evaluate the situation. Caten (**1972**) has suggested 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. The defense may be providing only partial protection in *C. parasitica,* with differences at a few, or certain, gene loci allowing some anastomoses which do not lead to rapid cell death (Anagnostakis and Waggoner, **1981**; Bazzigher *et al.*, **1981**; Anagnostakis, **1984b**; Kuhlman and Bhattacharyya, **1984**; Kuhlman *et al.*, **1984**). It is clear, however, that whatever the hypovirulence determinants are in *C. parasitica,* they must be considered "infectious" in some sense because of their ability to move from one mycelium to another, perhaps in a very short time.

PROSPECTS

The phenomenon of hypovirulence is now reported to be widespread in Italy (Mittempergher, **1978**; Palenzone, **1978**; Turchetti, **1978**, **1982**; Bisiach *et al.*, **1985**; Falcini *et al.*, **1980**), has moved into Switzerland (Bazzigher *et al.*, **1981**), and has been successfully introduced into France (Grente and Berthelay-Sauret, **1978**; Grente, **1981**). In all of these cases, the presence of hypovirulent strains of *C. parasitica* is correlated with heavy callousing of the host bark in and around cankers, and survival of the trees in spite of infection (Grente, **1981**). The rate of the natural spread of hypovirulent strains through blighted European chestnut stands in France has been calculated as one to two meters per year (Grente and Berthelay-Sauret, **1978**; Kuhlman, **1978**; Shain, **1982**). The Italian chestnut trees are now "recovering" from blight and are again a source of timber, and nuts for domestic use and export (Grente, **1981**; Turchetti, **1982**).

In the United States, *C. parasitica* strains with European hypovirulence are successfully used for "biological therapy" but cannot be demonstrated to spread in a predictable way; and therefore cannot be considered a biological control. There is some evidence, however, that the native H strains discovered in Michigan, outside the natural range of American chestnut trees, are spreading through the groves of chestnut trees planted there by settlers (Fulbright *et al.*, **1983**; Garrod *et al.*, **1985**).

Introduction of hypovirulence determinants into *C. parasitica* strains eliminates their ability to complete sexual reproduction as females (Anagnostakis, **1984a**). Therefore, the dispersal of hypovirulent strains must proceed by conidial dispersal alone. Hypovirulent strains may survive saprophytically in the forest, in some undiscovered niche, but they probably cannot survive by infecting American chestnut trees. Inoculation of hypovirulent strains alone into *C. dentata* usually results in very small cankers, with expansion rates an order of magnitude lower than those for virulent strains (0.11 to 0.17 mm per day, compared to 1.0 to 1.3 mm per day) (Anagnostakis and Aylor, **1984**). The H strains may be eliminated as the host bark forms callous and is sloughed off. If, however, conidia of H strains reach a virulent canker and are of a v-c type that will allow anastomoses and cytoplasmic gene transfer, they may convert the resident virulent strain to hypovirulent (Jaynes and Elliston, **1980**; Kuhlman, **1983**; Kuhlman and Bhattacharyya, **1984**). How much of a reservoir of hypovirulence in what diversity of v-c must we have in a forest to start and continue the kind of biological control observed in Europe?

If hypovirulence does not spread and/or if we stop treating new cankers, trees will be rapidly killed. Much of the *C. parasitica* inoculum may then be lost due to competition with decay organisms in the dead bark. *C. parasitica* does not penetrate into the xylem and therefore will not remain in the standing wood, which may persist for 50 years after the bark is rotted away (Brewer, **1982**). As sprouts form from the tree roots, a new cycle of the epidemic may begin again. Such cycles begin slowly, and disease incidence then increases rapidly (Hebard, **1982**). In one small area in Connecticut where such a cycle was followed for four years, the number of v-c types in new cankers on 50 trees went from six in 12 cankers, to 48 in 267 cankers (Anagnostakis and Kranz, **1987**).

The virulent *C. parasitica* population is obviously limited by availability of live host sub-

strate. Let us consider situations in which the population might be limited by hypovirulence.

The best possible situation is that found in France and in Greece where clonal introductions have precluded sexual reproduction, and dispersal of virulent strains proceeds slowly (Xenopoulos, **1982**; Grente, pers. comm.). In France, hypovirulence dispersal and transfer proceeds almost as fast as virulent strain dispersal and infection. Grente (**1981**) says that they treat every canker they can reach in an orchard for three years (usually 10 trees per hectare per year), and then 5 trees per hectare are treated in each of the next two to three years. This effectively establishes hypovirulence in the population and the orchard is saved.

If sexual reproduction is possible but few v-c genes are present in the population, there will be little diversity in spite of recombination during sexual reproduction. With few v-c types in the population, hypovirulence should be able to spread, though more slowly than in an area with only one v-c type. Fulbright and his colleagues in Michigan are now collecting data on the spread of hypovirulence in their orchards. The spread is slow but measurable (Garrod *et al.*, **1985**). Very few data are available on the v-c diversity in Michigan, but Fulbright's conversion success so far in laboratory experiments suggests that there are few v-c types, or few conversion-compatibility types, present in the Michigan *C. parasitica* populations.

This may also be the case in Italy where sexual reproduction was observed early in the epidemic, and virulent *C. parasitica* spread at about 30 km per year (Shain, **1982**). Even though few v-c types may be present in Italy (Anagnostakis *et al.*, **1986**) it is difficult to understand how hypovirulence could spread through the *C. parasitica* population at almost the same rate as the virulent strains spread through the chestnut orchards (Turchetti, **1982**; Shain, **1982**). In the course of studying spread of hypovirulence near Milan (Bisiach *et al.*, **1985**) Bisiach and his colleagues have only rarely seen hypovirulence conversion incompatibility, and when pairing 60 to 70 virulent strains from one area with five H strains, only three combinations resulted in a clear barrage (Gobbi, pers. comm.).

Shain (**1982**) has considered all the data available and concludes that a carrier must be operating in Italy. Turchetti and Chelazzi (**1984**) have recently found that tree climbing slugs (gastropods, *Lehmannia marginata* Müller) effectively transport hypovirulent inoculum and feed on the fungal stromata in cankers. In many chestnut orchards in Italy the young trees are cut on a 7 to 20 year rotation (Elliston, **1982**) and movement of this material by people may also explain dispersal.

No data are available yet on the rate of hypovirulence spread in Switzerland. From Figure 6 in Bazzigher *et al.* (**1981**) it appears that hypovirulent strains then occupied about ¼ of the area of virulent strains. Since only a few v-c types have been found there (five common, seven rare) it will be very interesting to observe the progress of hypovirulence.

Finally, what are the chances for biological control of chestnut blight in the United States by hypovirulence? On the basis of the population data for the small plot in Connecticut mentioned above (Anagnostakis and Kranz, **1987**), the prospect is grim. Rapid population diversification and rapid spread occurred there early in the epidemic cycle, and little spread of hypovirulence has been observed so far, despite continued treatment of cankers.

There is one hope for biological control in such a situation. Hypovirulence was first observed in Italy (Biraghi, **1953**) and in Michigan (Brewer, **1982**; Fulbright *et al.*, **1983**) about 15 years after the virulent fungus arrived. In these places it is described as appearing in the new cankers formed in the second cycle of the epidemic. Perhaps hypovirulence confers some survival advantage on the strains of *C. parasitica* that it infects. The dsRNA in hypovirulent strains of *C. parasitica* may be viewed, for practical purposes, as a plasmid. As far as we can determine it has no "free state" but is part of the cells (Buck, **1980**). It has been suggested (Levin and Lenski, **1983**) that bacterial plasmids generally impose a high cost on their hosts in terms of growth rate. Therefore, for plasmids to be maintained in a population, in spite of all the selection against them, they must carry genes that (under at least some conditions) enhance the fitness of their immediate hosts or that of cells carrying that plasmid in the population at large. As long as there is a finite rate of segregation yielding plasmid-free members, the plasmid will not be maintained if it is simply selectively neutral and replicating with a high fidelity (Levin and Lenski, **1983**). The conidia produced by hypovirulent strains of *C. parasitica* usually include some without hypoviru-

lence determinants (10% to 20%, see Grente and Sauret, **1969b**). Nevertheless, hypovirulence is established and appears to be maintained in the *C. parasitica* population in Italy where it first appeared, and in France where it was introduced. Perhaps hypovirulence determinants do confer some as yet undetected advantage, and failure of spread in the United States so far has simply been due to the hypovirulent population being smaller than some necessary threshold size. If so, hypovirulent strains may slowly begin to predominate in the United States where we have introduced them and where they have appeared spontaneously.

Grente's success in bringing chestnut blight into balance in France shows us that this, and other introduced pathogens, might be controlled in the United States. It is clear that we are only beginning to understand the complex ecology of this system, and we must continue to study the problem from all possible angles hoping for that synthesis that allows rapid scientific progress to occur.

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