Pathogenicity and Sporulation of Normal and Diseased Strains of *Endothia parasitica* in American Chestnut

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ABSTRACT.— Normal strains (all lacking double-stranded RNA) and diseased strains (all containing double-stranded RNA) of Endothia parasitica were screened for pathogenicity and asexual sporulation in excised dormant American chestnut stems in the laboratory and for pathogenicity, asexual, and sexual sporulation in intact trees in the field. Representative strains were included from French, French-derived American, Italian, and native American collections. In excised stems, normal strains from all collections produced rapidly expanding cankers with abundant pycnidia and spore tendrils. Diseased strains from the French collection and American diseased strains derived from them were either nonpathogenic or produced small cankers that did not enlarge or sporulate. Diseased strains from the Italian and native American collections exhibited wider ranges of pathogenicity and capacity to sporulate. Cankers produced by diseased strains in excised stems and intact trees could be distinguished from those produced by normal strains by their smaller size, fewer pycnidia and spore tendrils, or both. Only 2 of 17 pathogenic diseased strains produced perithecia and ascospores under field conditions during the six months after inoculation, and these were produced in very low numbers compared with normal strains.

Four collections of normal and diseased strains of Endothia parasitica Murr.) P. J. and H. W. And., the chestnut blight fungus, are being studied at the Connecticut Agricultural Experiment Station. These include: 1) French strains, F, obtained from J. Grente, INRA, Clermont-Ferrand, 2) American strains, FA, derived from the French strains, 3) Italian strains, I, obtained from L. Mittempergher, University of Florence, and 4) native American strains, A, obtained from naturally occurring cankers on American chestnut (Castanea dentata [Marsh.] Borkh.)

In this and a related study, comparisons were made of the morphological characteristics and pathogenic and reproductive capabilities in chestnut of representatives from these collections. Strains were examined concurrently by Day and Dodds at the Connecticut Station for presence of double-stranded ribonucleic acid (dsRNA), a type of nucleic acid typical of many fungal viruses and consistently found in strains termed "hypovirulent" (Day et al., 1977). Two major objectives of these studies were: 1) to determine the degree to which the various diseased strains are debilitated compared with normal strains and 2) to provide an indication of their potential for spread in nature by means of spores.

DEFINITION OF TERMS

For the purposes of this study a number of terms (boldface) were adopted and defined as follows:

The pathogenicity of a strain of E. parasitica is its capacity to kill host tissue, i.e., to produce a canker. The degree of pathogenicity of a strain can be expressed as rate of canker expansion or canker area after given periods of time. Virulence is distinguished from pathogenicity by combining with pathogenicity the capacity to sporulate, both asexually and sexually.

Normal strains of E. parasitica are those with morphological and physiological properties characteristic of strains causing typical chestnut blight in the wild. When grown on Difco potato dextrose agar (20 ml per 10 cm diameter petri dish) at 20 C under a 16-hr photoperiod, strains classed as normal colonize the medium in 5-7 days, produce abundant white to cream-colored aerial mycelium, exhibit moderately pronounced radial striations (consisting of aggregates of parallel hyphae alternating with regions of less numerous hyphae), and produce orange, approximately hemispherical pycnidia devoid of surface ornamentation and scattered within concentric rings that correlate with photoperiod. In excised stems of C. dentata these strains are capable of sustained pathogenesis, Le., they breach barriers erected by the host in advance of them, and produce abundant pycnidia and spore tendrils, usually within a period of two or three weeks at 20 C. Under field conditions normal strains are capable of producing, in addition, abundant perithecia and ascospores. Normal strains are by definition fully pathogenic and fully virulent.

Abnormal strains are those with abnormalities in one or more of the characteristics listed above. This class of strains includes numerous subclasses as illustrated in Figure 1. Diseased strains are abnormal strains with reduced virulence. Diseased strains that contain cytoplasmically transmissible genetic determinants, such as dsRNA, whose presence is correlated with their diseased state, are termed hypovirulent. Strains with mutant nuclear genes which cause low virulence and dsRNA-containing strains with normal virulence, if such exist,

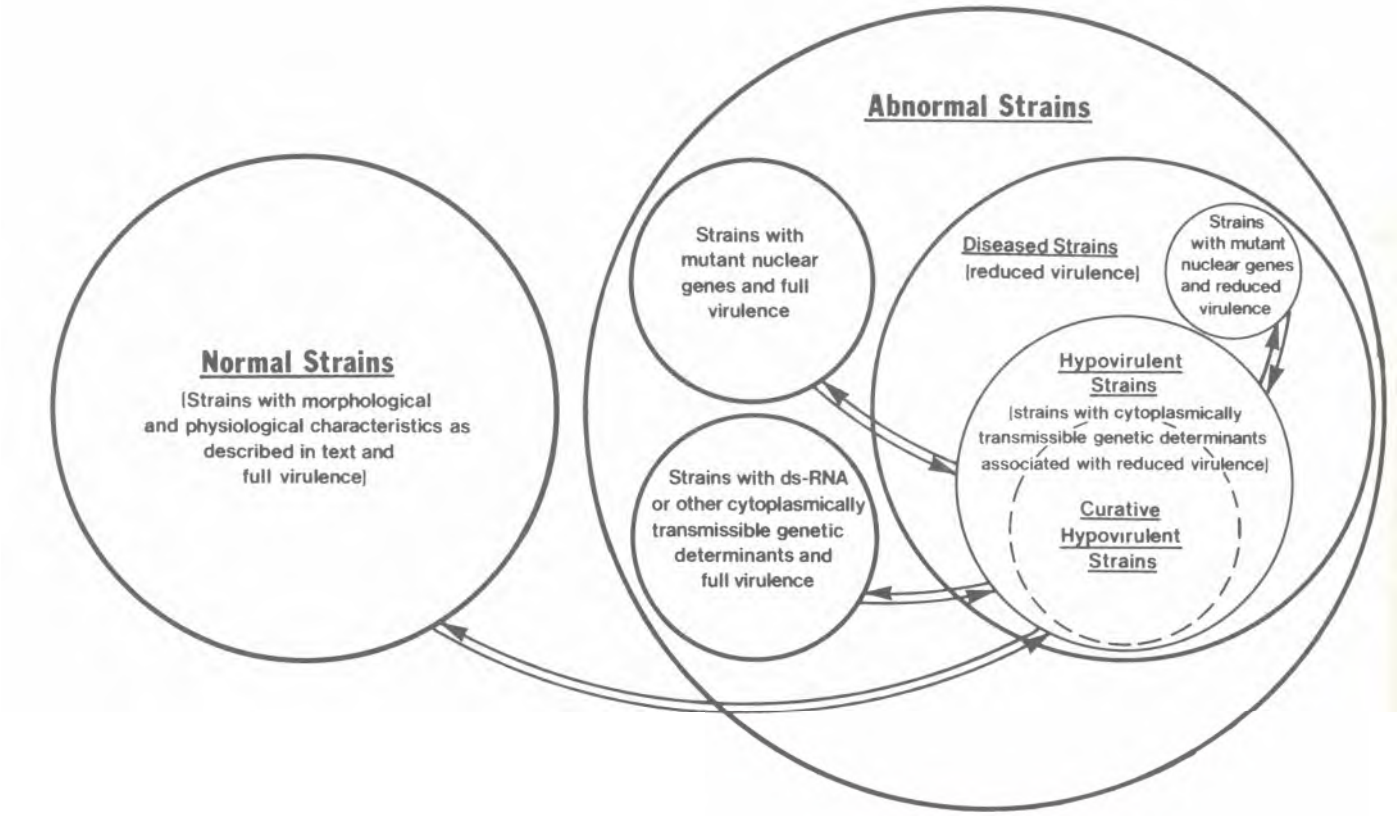

Figure 1. Hypothetical classification of strains of *Endothia parasitica.* Arrows leading to and from the hypovirulent class represent acquisition and loss, respectively, of cytoplasmically transmissible genetic determinants associated with hypovirulence.

are excluded from this class of strains. Hypovirulent strains may be fully pathogenic (at least in the short term) but are not fully virulent. Technically, for a diseased strain to be classified as hypovirulent, the transmissibility of the cytoplasmic determinant must be clearly demonstrated and its presence clearly correlated with the diseased state. To date this has not been accomplished with any of these strains but is a major objective of future studies.

Hypovirulent strains that transmit their cytoplasmic determinants to strains causing blight cankers, debilitating them, and thereby preventing tree death, are designated curative hypovirulent strains. This term is synonymous with "exclusive hypovirulent strains" (Grente and Sauret, 1969a). It should become evident from what follows that curative and noncurative hypovirulent strains, if the latter exist, would be difficult or impossible to distinguish in short-term experiments or with trees of small diameter.

LABORATORY DETERMINATION OF PATHOGENICITY AND CAPACITY FOR ASEXUAL SPORULATION

Preliminary experiments indicated that excised dormant stems of American chestnut are suitable for estimating pathogenicity of *E. parasitica* and capacity for asexual sporulation in the laboratory over a 5-6-week period. The stems used are at least 4 cm diameter with smooth, relatively thin bark, harvested between leaf drop and bud break, cut into 1.2 meter lengths, cut ends and branch stubs sealed with embedding wax, thoroughly scrubbed with a cheese cloth pad and plenty of water, dried, and stored until used in plasic bags at 4 C. Stems from different trees vary in susceptibility to *E. parasitica,* therefore all strains compared in an experiment are inoculated into stems from the same tree.

In the experiments reported here, 33 strains, including representative normal and diseased strains from each of the four collections, were inoculated into excised stems from each of nine trees. Each strain was inoculated into two sites on each tree, on opposite sides of the stem. A disk of bark and sapwood approximately 4 mm thick and 7 mm diameter was removed with a sharp cork borer, and this wound was inoculated with two 8 mm diameter plugs of agar cut from just within the advancing margin of colonies actively growing on Difco potato dextrose agar. The plugs were inserted with the mycelium facing inward and pressed into complete contact with host tissue using a flamed stainless steel spatula. Inoculated sites were covered with squares of masking tape to retard drying. The masking tape was removed after one week. Inoculated stems were incubated at 20 C under conditions of moderate humidity and a 16-hr photoperiod.

Canker length and width were measured and cankers examined for presence of pycnidia and spore

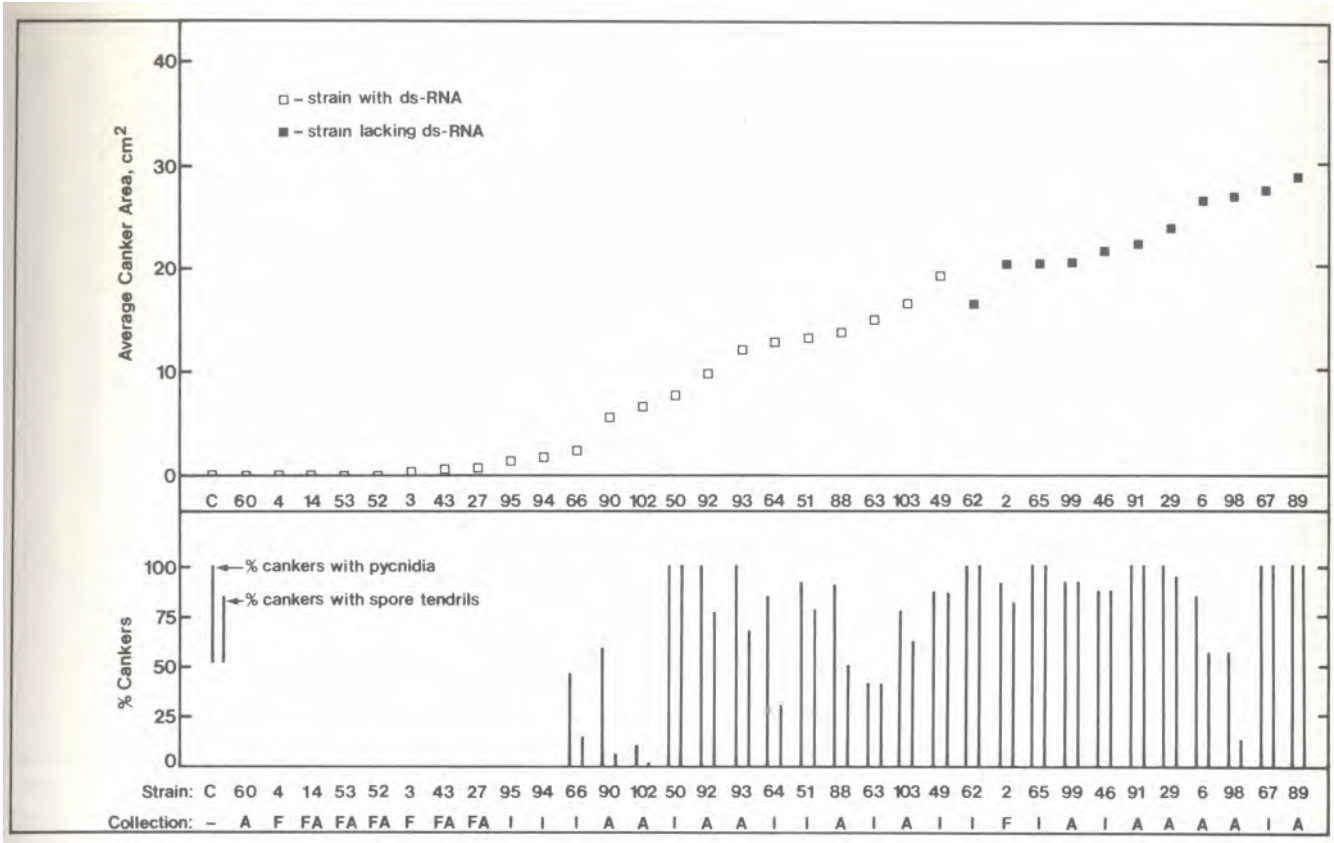

Figure 2. Pathogenicity and asexual sporulation of *Endothia parasitica* strains in excised American chestnut stems five weeks after inoculation. Each point is an average for 18 cankers. A, American; F, French; FA, French-derived American; I, Italian.

tendrils at 3- or 4-day intervals for 4-5 weeks beginning 7 days after inoculation. Results are summarized in Figure 2. Pathogenicity is expressed as average canker area in cm^2 five weeks after inoculation, corrected for area of the inoculation site. Strains are arranged from left to right in order of increasing pathogenicity.

It is evident that this collection of strains exhibits a continuum of pathogenicity. With one exception, average canker area for strains lacking dsRNA was larger than for strains containing dsRNA. However, a sharp break in average canker size between the two groups of strains was not observed. Five of the diseased strains were completely nonpathogenic, five produced small cankers that ceased expanding within two weeks after inoculation and did not sporulate, and the remainder showed various combinations of pathogenicity and ability to sporulate. Strains lacking dsRNA had a narrower range of pathogenicity and sporulation capacity. With two exceptions these strains produced abundant pycnidia and spore tendrils. The two exceptions, strains 6 and 98, are both methionine-requiring auxotropic mutants. Their methionine requirement may be responsible for their diminished capacity to sporulate. In accordance with the classification depicted in Figure 1, these strains have been transferred from the normal to the abnormal class of strains.

DETERMINATION OF PATHOGENICITY AND CAPACITY TO SPORULATE IN THE FIELD

Twenty strains of *E. parasitica,* including the 12 diseased strains found in laboratory tests to be capable of sporulating in *C. dentata* (strains 66-49, Fig. 2), six diseased strains not included in the above laboratory tests (strains 9,47,48,601, 901, and 120), and an American and an Italian normal strain (strains 29 and 46, respectively), were tested for pathogenicity and capacity to produce the asexual and sexual spore stages in *C. dentata* under field conditions. In mid-June, 1977, 40 trees were inoculated. Each strain was inoculated into eight trees ranging in diameter from 4 to 12 cm at a height of 1.4 meters. Four strains were inoculated into each tree, one on each of four sides. Inoculation sites were separated from each other vertically by approximately 30 cm. Inoculations were made as described for laboratory tests. To date, cankers have been measured and examined for pycnidia, spore tendrils, and perithecia at four-week intervals for six months. Results obtained through December, 1977, are summarized in Figure 3. Strains are arranged from left to right in order of increasing pathogenicity.

With two exceptions pathogenicities of diseased strains, as indicated by average canker area after six months, were markedly lower than those of normal strains. Twelve of the diseased strains

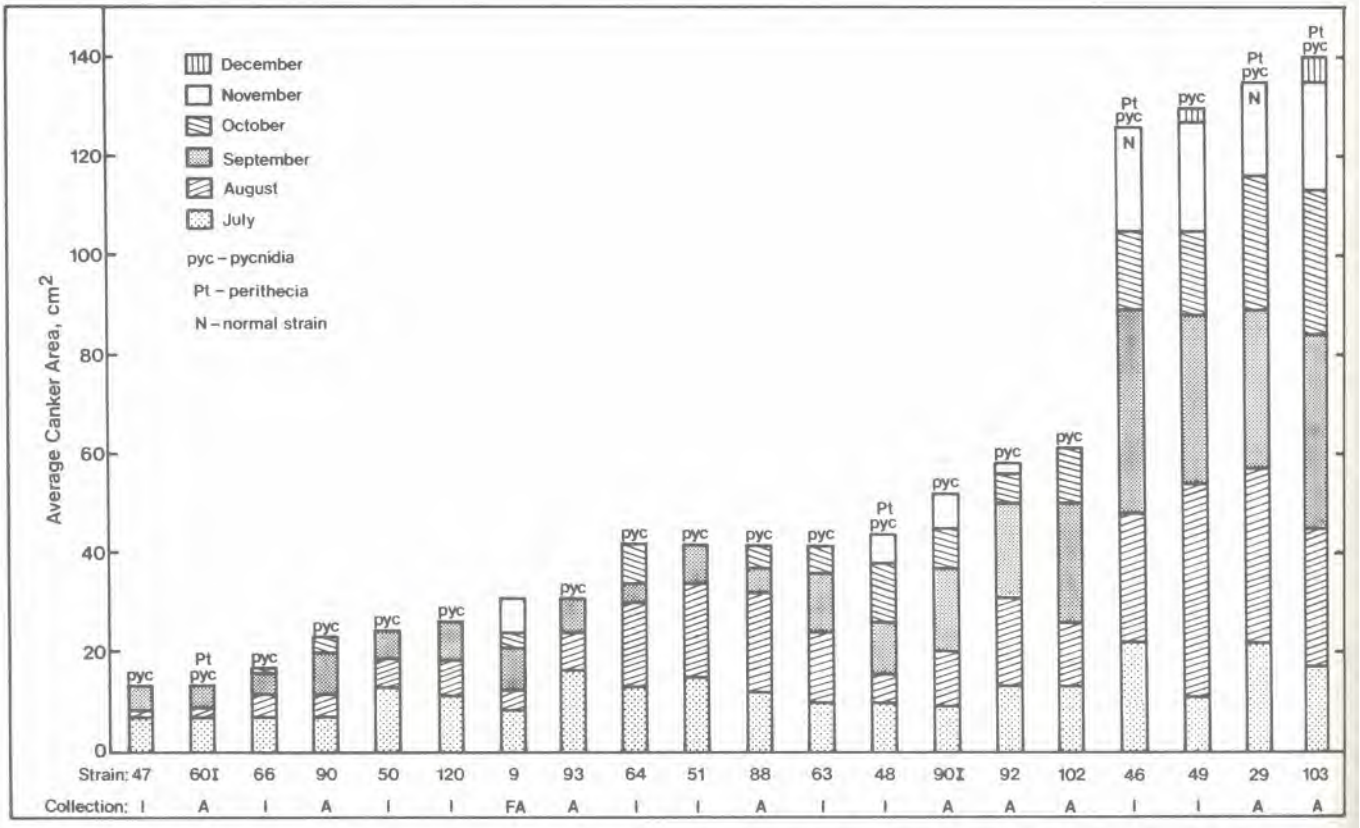

Figure 3. Pathogenicity and sporulation of *Endothia parasitica* in the field over a 6 month period (July-December, 1977). All data are averages for 8 cankers. A, American; F, French; FA, French-derived American; I, Italian.

appeared incapable of sustained pathogenesis, average canker area having stabilized after three or four months. Within this group, however, differences in pathogenicity were observed. Cankers produced by four of the diseased strains with low pathogenicity (strains 9,48,901, and 92) continued to enlarge slowly through the fifth month. Four strains, the two normal strains (29,46) and two diseased strains (49,103), produced cankers that expanded rapidly and continuously through the fifth month.

Pycnidia developed on cankers produced by all of the strains except strain 9, a French-derived American strain. The abundance of pycnidia varied from strain to strain. For all strains, spore tendrils were most numerous one month after inoculation. On subsequent observation dates tendrils were absent from cankers produced by all strains except strain 49. This peculiar strain continued to produce them in low numbers through the fifth month.

Perithecia were first detected three months after inoculation and were most abundant on cankers produced by the two normal strains and by strain 103. Only two of the strains with markedly reduced pathogenicity, strains 48 and 601, produced perithecia during the six months after inoculation and these were present in very low numbers. Small bark samples containing perithecia of these five strains were taken in November and examined in the laboratory. Perithecia were teased out of the host tissue and crushed to release asci and ascospores. In all cases the perithecia, asci, and ascospores appeared typical of *E. parasitica.*

DISCUSSION

Excised dormant stems of *C. dentata* provide a convenient means for estimating pathogenicity of *E. parasitica* and determining its capacity to produce the asexual spore stage in the host. In addition to convenience, they offer the advantage of permitting tests of pathogenicity during late fall and winter, a period when experiments with intact trees are impossible. The intact, physiologically active tree in its natural setting offers several advantages over excised dormant stems for assessing the virulence of strains of *E. parasitica:* it reacts more vigorously to infection, permits an assessment of the ability of a strain to produce the sexual spore stage, and allows experiments to be continued for extended periods, permitting detection of strains in which hypovirulence, or evidence of abnormality, is expressed several months after inoculation or in subsequent growing seasons.

Whether determined with excised stems or intact trees, diseased strains of *E. parasitica* exhibit a wide range of pathogenicity and capacity to sporulate in the host. The ranking of strains according to

level of pathogenicity was similar with both methods.

Ten of the diseased strains studied in the laboratory (strains 60-94, Fig. 2) were completely nonpathogenic or exhibited very low levels of pathogenicity. None of these strains produced pycnidia or conidia in the host. Absence of sporulation is characteristic of the French and French-derived American diseased strains studied in these experiments. The pronounced curative capacity commonly associated with hypovirulent strains is most easily demonstrated with strains of this type. Two strains from this group (27 and 43) were used in our early field tests of hypovirulent strains as biocontrol agents (Elliston and Jaynes, 1977). Although these strains exhibited a pronounced ability to arrest cankers caused by compatible normal strains and promote healing, no evidence of natural spread has been observed within plots treated with them. If ability to sporulate plays an important role in natural spread, spread could not be expected in these plots unless the disease agent within the diseased strains and transmitted by anastomosis to the strain causing the canker, infects the fruiting structures of the normal strain, and they then produce disease-carrying spores. This possibility, largely unexplored, will be investigated in future studies.

Results of pathogenicity tests and biological control experiments suggest that greatly debilitated hypovirulent strains may be excellent agents for controlling individual cankers and maintaining individual trees but are unlikely to lead to natural spread. They appear to be too debilitated.

Twelve of the diseased strains studied in laboratory and field tests (strains 66-49, Fig. 2) exhibited a range of pathogenicity and capacity to sporulate asexually in the host. In the field, most of these could be distinguished from normal strains on the basis of average canker size three months after inoculation. Six of them ceased enlarging three months after inoculation, five after four months, and four continued to enlarge at a slow pace. The two most pathogenic diseased strains (49 and 103) cannot be distinguished from normal strains on the basis of pathogenicity (rate and duration of canker expansion) over the time periods studied. However, strain 49 can be distinguished from normal strains on the basis of other abnormalities: predominately white colonies in culture, light-colored stromata in cankers, abnormally low numbers of pycnidia in cankers, extended production of spore tendrils in the field, and absence of perithecia in field tests. In a previous experiment, isolates taken during winter from the margins of cankers produced by strain 49 have exhibited greatly reduced pathogenicity; strains 94 and 95, Figure 2, are two such isolates. Cankers produced by this strain may well become distinguishable from those produced by normal strains during the coming growing season.

Strain 103, found in numerous tests to contain dsRNA and therefore thought to be diseased, is not easily distinguished from normal strains. Its cultural characteristics, pathogenicity, and ability to produce asexual and sexual spore stages in the host resemble those of normal strains. Several explanations are possible. This strain may be infected with a different fungal virus that does not cause debilitation of the kind associated with hypovirulence. If so, it is the first example encountered of a strain of *E. parasitica* with dsRNA and full virulence (Fig. 1). The dsRNA analyses could have given false results; this appears unlikely. Alternatively, the fungus may have lost its dsRNA upon subculturing. This too seems unlikely. The question will only be resolved by additional analyses. On the basis of information presently available, it cannot be considered hypovirulent.

The more pathogenic diseased strains that are capable of producing pycnidia and conidia in the host may be best suited for natural spread and effective biological control. This suggestion means that some sacrifice of host tissue would be necessary to provide the substrate for reproduction of these forms of the fungus. If strains of this type are required for natural spread, it would be unrealistic to expect that the immediate outcome of a successful biocontrol program will be canker-free American chestnut trees! The diseased strains with low pathogenicity but sustained pathogenesis, e.g., strains 9,48,901 and 92 (Fig. 3), may be the most desirable types for biological control, especially if they develop primarily in the outer layers of bark as described by Grente and Sauret (1969a) for hypovirulent strains in European chestnut *(C. sativa* Mill.). The persistence of sources of diseased strain inoculum for extended periods is believed to be correlated with successful biological control of blight in Europe (Grente and Sauret, 1979).

The possibility that the more pathogenic diseased strains have curative effects when inoculated into cankers caused by compatible normal strains has been largely unexplored. The previously held view that to be hypovirulent a strain must lead to rapid arrest of treated cankers is probably too restrictive. The more pathogenic diseased strains are probably curative but act more slowly and less conspicuously. If the infected tree is sufficiently large and the normal canker sufficiently small when treated, a more pathogenic hypovirulent strain might provide adequate control, i.e., preserve the life of the tree, and at the same time generate sufficient hypovirulent inoculum to aid in natural spread. The curative capacity of this type of strain would be difficult to detect in laboratory tests with excised stems, since this host material usually can be maintained for no longer than six weeks after inoculation. A period longer than one full growing season may be required for the curative effect to become evident under field conditions, particularly with diseased strains such as strain 49.

If the set of strains studied in these experiments is representative of those involved in spontaneous biological control in Europe, it appears unlikely that

the ascospore stage could play a significant role in natural spread. Only two of 17 pathogenic diseased strains studied under field conditions produced perithecia and ascospores during the six-month period following inoculation. These two strains produced very few perithecia compared with normal strains. It has not yet been determined if the ascospores produced carry dsRNA.

The only alternative inocula of diseased strains are conidia and mycelium. Some of these strains produce pycnidia and spore tendrils rather abundantly. However, none produce them as abundantly as normal strains.

Thus the diseased strains are at a competitive disadvantage on two counts: they produce fewer conidia than normal strains and few or no ascospores, the spore stage that is probably responsible for most long distance spread of the normal fungus here (Heald *et al.,* 1917) and Italy (Turchetti, pers. comm.). In addition, it is uncommon for all conidia produced by diseased strains to carry the disease agent (Bonifacio and Turchetti, 1973; Day *et al.,* 1977; Grente and Sauret, 1969b).

With the diseased strains at a severe competitive disadvantage with respect to their ability to produce propagules that could be spread by simple physical forces (wind, rain), one is almost forced to invoke the action of a vector if natural spread is to occur with the efficiency required for effective natural biological control. Spread of diseased strains (hypovirulent sensu Grente) in Italy has occurred without human intervention (Turchetti, 1979; Grente and Berthelay-Sauret, 1979). Perhaps an insect is involved that is specifically attracted to and feeds upon chestnut blight cankers. Evidence of feeding was observed in July and August on natural cankers and certain of the cankers produced by strains 29 and 49 in field plots from the experiment reported here. Thus, potential vectors for diseased strains exist in this country. They should be identified and their capacities to serve as vectors determined.

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