Epidemiology of Hypovirulence

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ABSTRACT.— Unless hypovirulence will spread naturally among virulent infections of *Endothia parasitica* on American chestnut it will have little or no significance as a biological control. The major constraints to natural spread include: 1) presence of vectors, 2) density of virulent infections, 3) vegetative compatibility, and 4) degree of hypovirulence.

The significance of hypovirulence as a biological control of chestnut blight in North America depends on its ability to spread naturally among virulent infections of American chestnut *(Castanea dentata* [Marsh.] Borkh.). To date we have not demonstrated natural spread of introduced hypovirulent strains in this country. However, the recovery of native hypovirulent strains of *Endothia parasitica* (Murr.) P. J. & H. W. And. that contain dsRNA from Michigan (Day *et al.,* 1977) suggests that hypovirulent strains both spread and maintain themselves on *C. dentata.* This paper examines some constraints to natural spread and compares the North American situation with what we know of the situation in Europe (see Grente and Berthelay-Sauret, 1979; Turchetti, 1979; Mittempergher, 1979.

MODE OF TRANSMISSION

From the work of Berthelay-Sauret (1973) and Van Alfen *et al.* (1975) it is now clear that hypovirulence is caused by a cytoplasmic determinant transmitted by hyphal anastomosis. This cell fusion creates a cytoplasmic bridge between infected hypovirulent and healthy virulent strains across which the determinant can move. A mounting body of evidence suggests that the determinant is a virus containing double-stranded RNA (Day *et al.,* 1977, Dodds, 1979) and that several forms of virus exist that may be distinguished by their dsRNA components, or by their effects on the colony morphology and degree of pathogenicity of their host fungus, *E. parasitica.* Transmission in the field can be readily effected by placing hypovirulent inoculum in holes cut in the bark at the edge of advancing lesions. Grente (pers. comm.) has stressed the importance of placing hypovirulent inocula closely spaced around the entire canker periphery to ensure that transmission occurs in all parts and is not restricted by dead or blocked regions of the virulent mycelium responsible for the canker.

Effective transmission can be verified by observing that the canker ceases to enlarge and that callus is formed by the host, and by isolating strains from the formerly virulent canker that are hypovirulent with respect to colony morphology, dsRNA content, and their behavior when reinoculated into stems (Elliston, 1979). In Connecticut, Jaynes and Elliston (1979) have observed control after spraying virulent cankers with suspensions of conidia of hypovirulent strains, suggesting that natural spread of hypovirulence by this spore form might occur. Grente (pers. comm.) has claimed that in Europe the healing cankers caused by hypovirulent strains on *C. sativa* Mill. are fissured, exposing superficial mycelium that has been prevented from penetrating more deeply by rapid cork formation. Fragments of host tissue bearing this mycelium can be carried by insects to other virulent infections.

The dispersal and rapid spread of virulent strains of *E. parasitica* in North America is considered to result principally from discharged ascospores which are airborne and can be carried for considerable distances. Day *et al. (1977)* examined cultures from single ascospores borne in perithecia formed on cankers that had been successfully treated with a hypovirulent strain. None of these ascospore cultures were hypovirulent or carried dsRNA. Since the perithecia could have formed on regions of the canker that had not been invaded by viruses, or could have existed as initials prior to treatment, these results are inconclusive. The finding that some hypovirulent strains that carry dsRNA form perithecia when inoculated alone to *C. dentata* (Elliston, 1979) should provide a test of whether viable ascospores are produced that contain dsRNA, and thus transmit hypovirulence. European experience suggests that hypovirulence is not transmitted by ascospores. Turchetti (pers. comm.) finds that in Italy hypovirulent cankers do not form perithecia but perithecia and ascospores are produced by virulent cankers. Grente (pers. comm.) has said that perithecia are uncommon in France.

POSSIBLE VECTORS

The early literature on chestnut blight in North America reflected the concern to determine the major mode of spread of virulent strains. Since *E. parasitica* invades wounds, some effort was made to find a vector, laden with spores, that also injured stems, allowing infection to take place. Certain insects were observed to feed on the stromata of cankers. For example Cerambycid beetles, *Amniscus (leptostylus) macula* (Say), were shown to eat *Endothia* pustules carried by pieces of bark in cage-feeding experiments (Craighead, 1912; Anderson and Babcock, 1913). Tests for viable conidia in the viscera and excreta of these beetles were negative. Craighead (1912) also recorded a Colydid beetle, *Synchita fuliginosa* Melsheimer, as eating *Endothia* pustules, stroma, and even conidial threads. However, since none of these vectors made wounds on otherwise healthy trees it was concluded that their role in dissemination was minor and that airborne ascospores were the most likely means of spread. In contrast, the spread of hypovirulent strains does not require wound inoculation but rather contamination of established virulent lesions with inocula capable of anastomosis and thus of viral transmission. From this point of view, insects that preferentially feed on conidial stromata are ideally suited since they are likely to carry virus from one canker to another by spores or mycelia adhering to their legs and mouthparts. My colleagues and I are greatly indebted to Dr. F. C. Craighead who wrote to us in 1977 with this suggestion and drew our attention to his early published work on insects associated with chestnut blight lesions.

Birds were also examined as potential vectors by shooting individual specimens, washing them in sterile water and either plating the washings or examining sediments after centrifugation (Heald and Studhatter, 1913). Large numbers of viable conidia were often recovered (Table 1). Birds such as the brown creeper *(Certhia familiaris* L.) could act as vectors, in a less direct fashion than insects feeding on cankers, since their feet and tail feathers could carry inoculum as they creep spirally up trees searching for insects in the fissured bark. Woodpeckers could be even more effective. Stewart (1912) suggested that blight spores are carried long distances by woodpeckers seeking borers in blight diseased trees. They "have a strong bill, sharply pointed for chipping and digging into tree trunks or branches for wood boring insects. The stiff tail is used as a prop" (Robbins *et al.,* 1966). Where beetle larvae are common in blight cankers they attract woodpeckers which would then carry spores and mycelia on their bills and tails to other cankers. Clearly, hypovirulent strains that produce large numbers of conidia will be best adapted to such methods of dispersal. Grente (pers. comm.) has observed that in France the B type hypovirulent strains he has used, which sporulate much less profusely than normal virulent strains, are spread in the form of mycelia in and on bark fragments carried by carpenter ants. Grente and Berthelay-Sauret (1979) have recorded that in France the rate of radial spread is of the order of 5-10 meters in five years.

The possibility of mass rearing insects that feed on blight cankers and releasing them, possibly after artificially contaminating them with hypovirulent inoculum, is complicated by the 1-2-year life cycles of the Cerambycid beetles identified in the early literature as vectors of *Endothia parasitica* (Welch, pers. comm.). Much more needs to be learned from field observation of vectors and of ways in which their dissemination of hypovirulent strains might be encouraged without detrimental effect on chestnuts or other components of their ecosystem.

Table 1

Pennsylvania birds shown to carry conidia of *Endothia parasitica*. Data of Heald and Studhalter (1913).

Species	Number of Birds Tested	Number Carrying *E. parasitica* Spores
Downy woodpecker (*Dryobates pubescens* L.)	16	13[a]
Sapsucker (*Sphriapicus varius* L.)	2	2
Brown creeper (*Certhia familiaris* L.)	2	1[b]
Nuthatch (*Sitta carolinensis* Latham)	2	1
Junco (*Junco hyemalis* L.)	2	1
Golden-crowned Kinglet (*Regulus satrapa* Lichenstein)	1	1

[a] Three birds shot two to four days after heavy rain carried from 109,000-750,000 conidia each.

[b] Bird shot four days after heavy rain carried 250,000 conidia.

DENSITY OF VIRULENT INFECTIONS

The determinant of hypovirulence, which we now believe to be a fungal virus, behaves as a parasite of *E. parasitica.* Its rate of spread will therefore depend on the density of its host. *E. parasitica* is in general well enough dispersed within the natural range of *C. dentata* in North America that its density is determined by the density of C. *dentata* stems. Most of these stems are sprouts surviving from the roots of trees killed earlier by blight. In Connecticut, occasional stems as large as 36 cm in diameter 1.3 m above ground may be found. However, stem density in most Connecticut forest plots is low, ranging from 25 to 75 stems 2.5-10 cm in diameter (1.3m) per ha, in areas where chestnut is common. In contrast, in areas of West Virginia that were recently clearcut, MacDonald and Double (1979) have established plots of regenerating *C. dentata* with densities approaching several hundred stems per ha.

In some Connecticut forest plots canker density approaches an average of two or more per stem. Natural spread of hypovirulent strains will be most easily observed in dense stands in which chestnut growth has been encouraged by either clear-cutting or selective cutting, possibly supplemented with fertilization and irrigation. The establishment of dense plots as "hypovirulent infection centers" will be an important next step in the evaluation of this method of biological control.

European experience indicates that stand density plays an important role. In Italy and France, chestnut orchards consist of pure stands of trees often with branches in contact. In Italy, hypovirulence has spread unaided by man in productive and abandoned chestnut orchards (Turchetti, pers. comm.), and also in wild stands (Mittempergher, 1979). A survey of cankers on *C. sativa* in 1977 by Palenzona (Grente and Berthelay-Sauret, 1979) showed that the incidence of hypovirulent strains in the Piedmont region of north Italy ranges from 60-90 percent.

VEGETATIVE COMPATIBILITY

Hypovirulence is transmitted from one mycelium to another through points of anastomosis that establish cytoplasmic continuity. If the mycelia differ genetically, and exchange of nuclei follows anastomosis, a heterokaryon may be formed, or if they carry different cytoplasmic elements, a heteroplasmon. In fungi, hyphal anastomosis, and its consequences, are subject to genetic controls that restrict fusion and establishment of bridges to strains that share common alleles at one or more loci. These controls govern what is called heterokaryon or vegetative incompatibility (Anagnostakis, 1977). *Endothia* has such controls and they interfere with transmission of hypovirulence among different strains. Transmission occurs most readily between strains that belong to the same compatibility group. However, it does occur at a lower frequency, between strains that are not compatible.

Grente (pers. comm.) has reported some 50 compatibility groups in *E. parasitica* from studies involving several hundred tests. A set of white hypovirulent tester strains was paired with the unknown virulent strain to see which tester converted the virulent to hypovirulent. The test was carried out on cellophane over an agar medium. Anagnostakis (1979), using a different test, reported 46 compatibility groups among some 200 isolates, mostly from North America. Her tests paired virulent strains on agar medium and recognized incompatibility reactions that were less clear with hypovirulent strains. Genetic controls of vegetative incompatibility in some ascomycetes may involve ten or more different genetic loci. At least six appear to be functioning in *E. parasitica* (Anagnostakis, 1977). It is very likely that incompatibility due to heteroallelism at some of these loci creates a more effective barrier to virus transfer than heteroallelism at others. However, a cytoplasmic bridge that is quite short lived may be sufficient for infection to occur, albeit at a low frequency.

Grente's test method has an advantage in generating new hypovirulent strains that are isogenic with each unknown and which can then be used for field inoculation in areas where the unknown is prevalent. However, the method may restrict field release to one or a few kinds of hypovirulence when it might be more advantageous to release mixtures of hypovirulent strains (see below).

Grente and Anagnostakis differ in the amount of variation they report among collections of *E. parasitica* from the field. Grente (pers. comm.) finds that in France one compatibility group is characteristic of an area that includes a number of infected trees. Anagnostakis (1979) has found up to ten groups present on one *C. dentata* stem. MacDonald and Double (1979) have made similar observations in West Virginia. This is not surprising since in North America the role played by ascospores in dispersal will ensure great variation in incompatibility types, whereas in France perithecia are reportedly uncommon.

For the time being the most promising method of release in North America appears to be the use of mixtures of hypovirulent strains that include several different compatibility groups (Jaynes and Elliston, 1979).

DEGREE OF HYPOVIRULENCE

Elliston (1979) has shown that hypovirulent strains may vary considerably in their pathogenicity when inoculated alone. Hypovirulent strains that are nonpathogenic may be expected to effect rapid cures in tests but will contribute little or no inoculum for infection of other cankers. For this reason there seems to be little point in releasing them in infection centers for biological control. However, such strains occur in nature (e.g. strain 60 from Michigan) and so presumably are either maintained, possibly as saprophytes on dead chestnut stems, leaves, and other litter, or they are generated as part of the variation shown by hypovirulent strains. At the present time we know very little about the molecular biology of hypovirulence, how variants are generated, or about how they interact with different virulent strains of *E. parasitica*. In the meantime, an effective strategy may be to use a wide range of hypovirulent types as mixtures in infection centers and observe which ones predominate. An evolutionary trend may work in our favor. All parasites eliminate their hosts, and also themselves, if they are too effective. Hypovirulence may saveE. *parasitica* from completing the destruction of C. *denata* and itself but, in doing so, natural selection will ensure propagation of hypovirulent forms that are also not self-destructive.

There are several important practical consequences from the balanced control situation implied above. First, we will have to accept the degree of canker development that will occur with effective and rapidly spreading hypovirulent strains. Observations of the site in Michigan (Day *et al.*, 1977) shows that the trees are chronically infected and that their growth and form are distorted by cankers

(Anagnostakis and Elliston, pers. comm.). However, many of the trees are alive, ungirdled, and capable of growth and reproduction. The Michigan trees may be atypical and represent an extreme. Certainly the slow growing, superficial, hypovirulent cankers described by our European colleagues on *C. sativa* appear to be less destructive. Thor (1979) has stressed the importance of breeding resistant trees by intercrossing native C. *dentata* survivors and selecting for blight resistant progeny. This technique, advocated for crop plants (Robinson, 1976), can be very effective but requires a number of generations of selection to accumulate the many genes of individually small effect that are required. In the meantime, as Thor points out, hypovirulence may tend to be confused with resistance, as it was by Biraghi (1953) who originally discovered it. Grente (pers. comm.) has stressed that the success of hypovirulence in Europe depends on the ability of *C. sativa* to resist penetration and restrict the development of hypovirulent mycelium to the outer layers of bark by formation of an effective wound periderm. Ability to form an effective necrophylactic (wound) periderm is a feature of most woody plants (Mullick, 1977). Although response to a given hypovirulent strain may vary among species of *Castanea,* the responses of *C. dentata* or *C. sativa* may also vary with different hypovirulent strains. Selection of the most effective hypovirulent strains will have to take account of the kind of host wound response they induce.

FUTURE PLANS

The use of hypovirulent strains to test biological control of blight in North America still requires observance of plant quarantine regulations. For the time being we will continue this practice and keep records of where exotic hypovirulent stocks are being released. All of our tests in Connecticut on native woody plants and field experience so far indicate that there is no danger from releasing exotic hypovirulent isolates. As testing increases, and more hypovirulent isolates are used, the regulations may become an impediment to progress.

For the time being it seems most efficient if testing and release is left to state agricultural experiment stations, universities, and U.S. Forest Service laboratories. We should soon be in a position to recommend mixtures of markedly hypovirulent strains to cure cankers on specimen trees. In several years time we should be able to judge the best methods for large-scale release and to suggest the means for accomplishing a self-perpetuating biological control that could one day, perhaps not in our lifetime, restore the American chestnut.

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