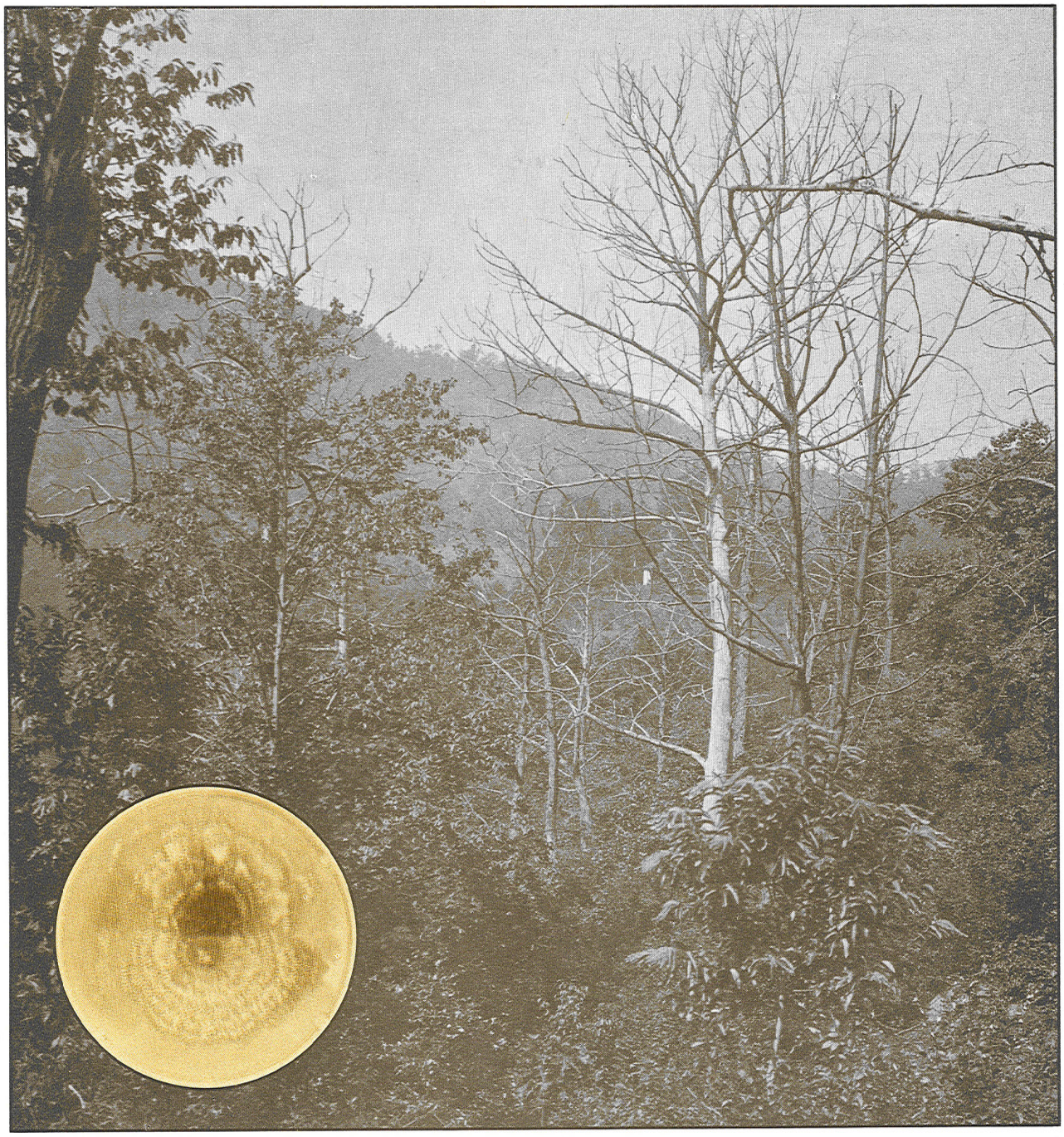
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Proceedings of the USDA Forest Service American Chestnut Cooperators' Meeting



Cover
Biological control of chestnut blight on American chestnut sprouts in the forest may result from the interaction of hypovirulent with virulent strains (inset) of *Endothia parasitica*.

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FOREWORD

The American chestnut was an important tree in the history of the United States. The fruit of chestnut was not only important to man and his domesticated animals but to the wildlife of the eastern forests. The tree comprised over 25 percent of the eastern hardwood forest and its natural range included at least 200 million acres. On good sites, the tree often grew 1 inch in diameter each year, faster than any of its associated hardwoods. Its ability to grow on poorer sites and produce a straight, clear bole made it an even more valuable species. Chestnut timber was of superior quality and durability having been used for fence posts, rails, furniture, paneling, and construction. Nowhere in the eastern forests is there as much public sentiment for a tree as in the Appalachian region where the value of the chestnut will long be remembered.

Recently there has been considerable renewed interest and optimism for the American chestnut tree. In Europe, the chestnut trees are recovering from chestnut blight. European researchers believe that hypovirulence, less virulent strains of the chestnut blight, is the explanation for this recovery. In the United States, hypovirulence has the potential to biologically control this disease, but there are many problems to solve and characteristics to be understood before hypovirulence can realistically be used as a means to allow the American chestnut tree to survive.

In January 1978, an American Chestnut Symposium was held in Morgantown, West Virginia. The Forest Service held its first cooperators' meeting at Pipestem, West Virginia in January 1980. Papers and abstracts of talks given at both meetings were published. During January 1982, a second Forest Service cooperator meeting was held at Morgantown, West Virginia. All cooperators funded by the Forest Service summarized the status of their research programs, including what they are doing, what they have found out, what problems have occurred, and future plans. The second aspect of the meeting involved specific research reports by many of the participants including research not funded by the Forest Service conducted at the Connecticut Agricultural Experiment Station, Michigan State University, and Western Michigan University. A third portion of the meeting involved a group discussion of the current status of five selected research topics relating to hypovirulence. Before each discussion, a discussion leader presented a state-of-the-art paper summarizing available, pertinent information. The discussion topics were vegetative compatibility, cultural studies, host-parasite interactions, molecular aspects, and dissemination of hypovirulence. Following the discussion topics, David Houston led a group discussion in an effort to establish priorities for the chestnut blight research program.

Through cooperative efforts of groups such as the universities, state and federal agencies, researchers hope to solve the mystery of the chestnut blight and provide ways for the American chestnut tree to regain some of its importance in the eastern hardwood forest.

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HYPOVIRULENCE IN ENDOTHIA PARASITICA AND SUGGESTED PROCEDURES FOR ITS DETECTION AND ANALYSIS

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ABSTRACT.--*Concepts of normalcy, virulence, and hypovirulence in Endothia parasitica are presented. Normalcy is defined by the characteristics of a set of four strains (standards) taken from the American and Italian populations of the fungus. Normal virulence is defined by the pathogenicities and reproductive capacities of the standard strains in American chestnut. Hypovirulence is then defined simply as a condition of subnormal virulence. Distinctions are made between two types of hypovirulence, cytoplasmic (CH) and nuclear (NH). These concepts are illustrated with examples of simple and complex hypovirulent strains from the North American and Italian populations of the fungus. Procedures are suggested for the detection and detailed analysis of hypovirulence in individual strains.*

Introduction

What is hypovirulence in the chestnut blight fungus, *Endothia parasitica*? Unfortunately, no precise, operational definition has been published. A narrow concept of hypovirulence is used in most chestnut blight literature and applies only to strains which have low or no pathogenicity and high curative capacity. These strains have been called curative hypovirulent strains to distinguish them from other types of hypovirulent strains (Elliston 1978), but here they will be referred to as 'H' strains. When an 'H' strain is inoculated into healthy bark of European or American chestnut, *Castanea sativa* and *C. dentata*, respectively, it invades, colonizes, and kills little if any tissue. Furthermore, when inoculated into an active blight canker, it rapidly arrests canker development and permits wound closure to proceed. These two properties distinguish 'H' from 'virulent', i.e., wild type or normal strains as clearly as white can be distinguished from black.

In southern Europe, the natural recovery of European chestnut from chestnut blight, which has occurred over the past 30 years, has been attributed to the appearance and natural spread of 'H' strains (Grente and Sauret 1969a; Bonifacio and Turchetti 1973; Mittempergher 1979; Bazzigher et al. 1981). Recovery has been accompanied by a reduction in canker incidence and changes in canker morphology (Grente and Berthelay-Sauret 1978; Biraghi 1950;

Turchetti 1979). The fungus in the abnormal cankers grows more superficially, fruits less abundantly, and stimulates the vascular cambium to lay down additional layers of bark and wood cells, giving the cankers a more or less swollen appearance (Biraghi 1953).

Other 'H' strains of the fungus have been discovered in abnormal cankers on American chestnut trees in areas of western Michigan (Elliston et al. 1977), where natural recovery also is occurring (Brewer this proceedings; Weidlich et al. this proceedings), and on isolated trees in Tennessee (Elliston et al. 1979) and Virginia (Elliston and Kuhlman unpublished data). Presumptive 'H' strains also have been isolated from abnormal cankers on American chestnut trees in other locations within the tree's natural range (Jaynes and Elliston 1982). The elements responsible for the recovery phenomena occurring on the two continents may be manifested in these strains.

Investigators have sought simple, reliable indicators of 'H' to simplify the tasks of detecting these strains in natural populations and studying them in the laboratory and field. Even though deeply pigmented 'H' forms occur (Grente and Sauret 1969b) and have been isolated from nature (Bonifacio and Turchetti 1973), it has been argued that 'whiteness' or slow development of color in culture is a reliable indicator of 'H' (Van Alfen et al. 1978). Double-stranded ribonucleic acid (dsRNA) in the 'white' European 'H' strains and their American derivatives and its apparent absence from 'virulent' European and American strains (Day et al. 1977) suggested that dsRNA might be a reliable indicator of 'H'. However, subsequent studies of a large collection of European and American dsRNA-containing strains (Elliston 1977; 1978 unpublished data) negated 'whiteness' in culture and presence of dsRNA as reliable indicators of 'H'. Wide ranges of cultural abnormalities, levels of pathogenicity, and fruiting capacity occur among these strains. In contrast, characteristics of dsRNA-free European and American strains are much more consistent, as had been reported by earlier investigators (Shear et al. 1917). Many of these observations have been confirmed by Willey (1980). Clearly, a continuum of virulence now occurs in the chestnut blight fungus in nature. We do not yet fully understand what elements are responsible for this virulence continuum, nor do we know how much each element contributes to the recovery phenomena now occurring in southern Europe and western Michigan. The preoccupation with 'H' strains, evident in much of the literature on hypovirulence, may blind us to the possibility that other, less abnormal forms have significant roles. Although curative capacity can be demonstrated most easily with the most debilitated dsRNA-containing strains, these strains could be end products rather than key components of these systems (Elliston 1982). It is possible that other *Endothia* species or even unrelated genera are principal donors of the elements for reduced virulence in *E. parasitica* (Elliston 1982).

A more general concept of hypovirulence may lead to a fuller understanding of the recovery phenomena we are studying. An operational definition of hypovirulence is needed if the revised concept is to be useful. This paper is an attempt to satisfy these needs.

A Revised Concept of Hypovirulence in *Endothia parasitica* and Associated Definitions

The literal definition of hypovirulence probably is most appropriate because it is simple, logical, and has the widest application. The prefix hypo, meaning less than the ordinary or norm (Websters New Collegiate Dictionary 1959), modifies virulence, meaning the disease-producing capacity of an organism (Steen 1971). Therefore, hypovirulence literally means any state of disease-producing capacity less than the norm. This definition merely sets apart all subnormal states of virulence from the normal state; it implies nothing about cause.

This definition of hypovirulence is meaningless without an operational definition for normal virulence, and normal virulence cannot be defined without operational definitions for virulence and normalcy. Many pathologists define virulence as the degree or measure of pathogenicity (Ainsworth 1961; Anonymous 1968; Wood 1967). Pathogenicity can be defined as the capacity to infect, colonize, and disrupt or kill host tissue. Steen's definition of virulence (Steen 1971) offers greater latitude than the common definition. The disease-producing capacity of an organism, in its broadest sense, includes both pathogenicity and reproductive capacity. Reproductive capacity is the pathogen's capacity to produce propagules adapted for dissemination from infected to uninfected tissue within host plant and from individual to individual within a population of the host. This concept of virulence provides the additional criteria needed to distinguish some of the less debilitated strains of *E. parasitica* from normal strains (Elliston 1978). Virulence in *E. parasitica* then, has four measurable components: amount of bark surface area colonized, depth of infection, number of mature pycnidia, and number of mature perithecia at one or more specified intervals after superficial inoculation of American or European chestnut. Depth of infection is included because strains that penetrate rapidly from the outer layers of bark to the vascular cambium and sapwood are more damaging, and therefore more virulent, than strains that colonize the same volume of bark tissue in the same time interval but grow more superficially. Assessment of both pycnidium and perithecium production is included because strains capable of producing both conidia and ascospores have greater disease-producing capacity than strains capable only of producing conidia. Although precise measurements of depth of infection and numbers of mature fruiting bodies would require extensive destructive sampling, rough estimates of these might suffice in most situations.

Normal strains of the chestnut blight fungus will be defined as those which are homokaryotic, i.e., have one type of nucleus, are free of infectious agents, and have characteristics typical of the species (Elliston 1978). This definition has meaning only if a standard set of strains is designated and used for comparison, and these strains are in their normal, vigorous physiological state when used. Degree of vigor usually is evident from cultural characteristics if one has had sufficient experience with the fungus. If in comparative tests a strain differs significantly from the standard strains in one or more characters, it is abnormal with respect to those characters. When that character is virulence, strains not significantly different from the standards have normal virulence, those with significantly lower virulence are hypovirulent, and those with significantly higher virulence are hypervirulent. A strain can have normal virulence and be abnormal in one

or more other characters (this would be an abnormal strain with normal virulence). Also, normal strains in culture often assume one of several transient, physiologically abnormal states in which their virulence is subnormal (these would be normal strains with transient hypovirulence).

These definitions apply at the species level. In practice, analysis of hypovirulence begins with individual strains taken from the natural population. Thus, there also is a need for a concept of normalcy at the level of the individual strain. This is especially true when a strain is encountered that is atypical of the species. Two types of strains can be distinguished: simple strains, which are homokaryotic, and complex strains, which are heterokaryotic, i.e., have two or more types of nuclei. The 'normal' state of a simple strain will be defined as that in which it is free of infectious cytoplasmic agents and growing most vigorously. Normal virulence for such a strain is its level of virulence when in this state. The strain is hypovirulent with respect to its normal state when its virulence is significantly lower. These definitions apply to complex strains only if the strain is stable. Otherwise these states cannot be defined. All states of an abnormal strain could be hypovirulent with respect to the standard normal strains.

Distinctions between types of hypovirulence are needed and might best be founded on whether the genetic information that determines them is nuclear or cytoplasmic. Nuclear hypovirulence (NH) is due to nuclear genetic determinants (NH agents) and might be caused by mutant nuclear genes, genes brought together in heterokaryons, hybrid nuclei, or extrachromosomal genetic determinants that reside in nuclei. Nontransmissible hypovirulence (Van Alfen this proceedings) may be a synonym for NH. Cytoplasmic hypovirulence (CH) may be conditioned by agents (CH agents) such as viruses and virus-like pathogens, plasmids, and organelles, such as mitochondria, that carry genetic elements. Since many of these agents also fit the definition of a hyperparasite, CH often can be viewed as a consequence of hyperparasitism, or more properly, hyperpathogenism. Synonyms that have been used for CH include 'exclusive' hypovirulence (Grente and Sauret 1969a), contagious hypovirulence (Grente 1975), transmissible hypovirulence (Van Alfen et al. 1975), infectious hypovirulence (Kuhlman 1980), and hypovirulence (Jaynes et al. 1976; Day et al. 1977).

It is probable that in some strains NH and CH agents act together to reduce virulence (Elliston 1982; unpublished data). The intensely pigmented strains from Europe, the 'JR' type of Grente and Sauret (1969b) and the 'P' type of Bonifacio and Turchetti (1973), behave as if they have abnormal nuclear conditions and contain virus-like CH agents (Elliston unpublished data). Rare, intensely pigmented single conidial isolates which are free of CH agents have been obtained from these strains. They fail to transmit CH agents into compatible normal strains unless they are first allowed to interact with compatible strains containing these agents. The subnormal virulence of the slow-growing, white European CH strains may be due to heterokaryosis and infection with virus-like CH agents. These strains typically yield complex mixtures of single conidial isolates. These include: typical normal; slow-growing, intensely pigmented; highly variable, unstable, slow-growing white; and fast-growing white types. The variable, slow-growing 'white' types, like the parent strain, probably are heterokaryons infected with more than one virus-like CH agent. Another strain, EP-405, from central Italy behaves as if it

contains at least three types of nuclei, based on the pattern of segregation of its conidia and the behavior of each of the types.

In Figure 1, several hypothetical strains are used to illustrate many of these concepts.

Suggested Procedures for Detection and Detailed Analysis of Hypovirulence

The detailed analysis of a strain for hypovirulence usually begins by comparing its cultural characteristics with those of two or more standard strains of *E. parasitica*. Strains EP-155, 408, 421, and 523, selected from the 22 dsRNA-free strains used in the study of selected CH agents (Elliston this proceedings), are now being used as standards in this laboratory. The origins of these four strains are given in Table 1 of that paper. Cultural characteristics are determined on 100 x 15 mm plastic disposable petri dishes containing 20 ml of Difco PDA amended with 0.1 g L-methionine and 0.1 mg biotin per liter (PDAmb). Preparing and dispensing this medium with a model AS-3 agarmatic bench-top agar sterilizer fitted with a model M1062 dispensing pump helps ensure uniformity. Inoculum for determining cultural characteristics consists of 7 mm diameter plugs of mycelium and agar cut with a sharp, sterile cork borer from the advancing margins of actively growing, vigorous colonies. The plugs are oriented with the mycelium side up. Three replicate plates of each strain are each sealed with a layer of parafilm and incubated with a 16 hr photoperiod at 20 C, 75 cm beneath banks of fluorescent lights spaced 25 cm apart. Cultures are examined when 7- to 9-days old. If the cultural characteristics of the test strain(s) are consistently different from those of the standards, the analysis proceeds. If the results are inconclusive, the comparative experiment is repeated. If the test strain is indistinguishable from the standards, it probably is normal. If its virulence is normal and it lacks detectable dsRNA, it would not be studied further.

If a strain has abnormal cultural characteristics, single conidial isolation experiments are conducted to determine if more than one type of single conidial isolate (SCI) is obtained. In each experiment, all germlings from two plates of complete medium (Puhalla and Anagnostakis 1971) containing approximately 50 germlings each, are transferred, cultured, and compared with standard normal strains and the parent strain. If only one type of isolate is obtained, the parent strain either is a mutant, a member of another species, or a CH strain in which the CH agent enters most of the conidia it produces. The SCI experiment would be repeated. If the same results are obtained, the strain probably would be tested for virulence in American chestnut, presence of dsRNA, transmissibility of the abnormality directly or indirectly to the standard normal strains, and capacity to mate with mating type testers of *E. parasitica* (Anagnostakis 1979), and, if perithecia are produced, ascospore progeny would be examined for the pattern of segregation of morphological types. The course of further analysis would depend upon the outcome of these tests.

If in the SCI experiment more than one isolate type were obtained, isolates representing each type would be retained and tested for virulence, stability in SCI experiments, presence of dsRNA, transmissibility to the most normal appearing type of SCI, and transmissibility directly or indirectly to the standard normal strains. If one of the SCI types is stable in further SCI

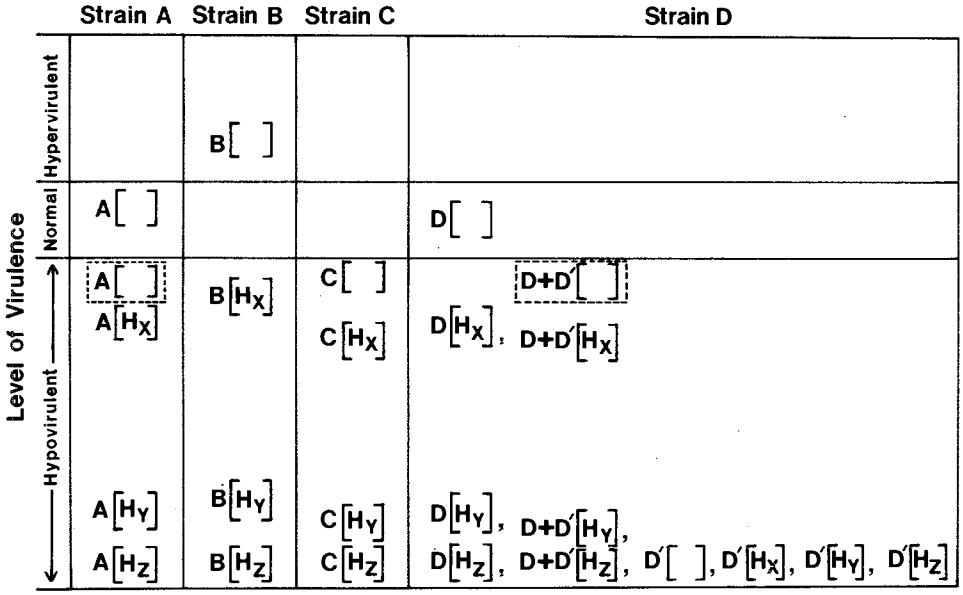


Figure 1. Four hypothetical strains of *Endothia parasitica* which illustrate many of the concepts discussed in the text. Each strain is presented in a variety of states, each having a different level of virulence. Each state is represented by one or more uppercase letters, A, B, C, D, or D', which represent different genotypes, followed by a set of brackets containing information about the state of the cytoplasm. A single letter signifies a homokaryon; more than one letter signifies a heterokaryon. Empty brackets indicate normal, uninfected cytoplasm. Symbols H_X, H_Y, and H_Z represent three different virus-like cytoplasmic hypovirulence (CH) agents which, when present in a strain, have mild, moderate, and severe effects on virulence, respectively. Only single infections are shown. Sets of symbols enclosed by dashed lines represent the strain in a state of transient hypovirulence. The normal level of virulence for the species is estimated using the four standard strains. Strain A, when uninfected with CH agents, has normal virulence, i.e., virulence typical of the species as estimated with the standards. It is shown in a state of transient hypovirulence and in three states of cytoplasmic hypovirulence. Strain B, when uninfected, is hypervirulent, i.e., more virulent than normal. It displays nuclear hypervirulence. It too is shown in three states of cytoplasmic hypovirulence. Strain C, when uninfected, is hypovirulent, i.e., it is less virulent than normal. It displays nuclear hypovirulence. When strain C is infected with one of the CH agents, it is in a state of cytoplasmic hypovirulence. Strain D is a complex strain containing two types of nuclei, D and D'. An isolate of strain D containing only the D type nucleus, and having uninfected cytoplasm, has normal virulence. When such an isolate is infected with a CH agent, the strain has cytoplasmic hypovirulence. An isolate containing only the D' type nucleus has severe nuclear hypovirulence. Its level of virulence is the same whether or not it is infected with CH agents. The heterokaryon, D+D', is unstable in the absence of CH agents. The normal nucleus and the CH agent largely determine the level of virulence of the heterokaryon.

experiments, lacks detectable dsRNA, and has some resemblance to typical *E. parasitica*, it is assumed to be the normal state of the strain. Its virulence is compared with that of the standard strains to determine if it is typical of the species.

The number of distinct abnormal SCI types obtained from the parent strain provides a clue to the number of elements present. If the parent strain consistently yields only two distinct SCI types, one of which resembles the parent and the other is dsRNA-free and has normal or near normal cultural characteristics, the parent probably contains a single agent conferring the abnormality. If the abnormality can be transmitted from the parent strain to the most normal SCI type, the agent probably is a cytoplasmic determinant. If the parent strain and the abnormal SCI type like it contain dsRNA with the same pattern, the parent probably is a CH strain containing one virus-like CH agent. If the abnormality and dsRNA are transmissible to one or more of the standard normal strains, this conclusion almost certainly is valid. The effect of the CH agent on the cultural characteristics and virulence of the standard strains can then be determined and compared with those of cultures of standard strains containing other CH agents.

If more than two SCI types are obtained from the parent strain, more than one agent is present, and the analysis becomes more involved. The parent strain could be a simple strain containing two or more CH agents or a complex strain containing two or more types of nuclei and one or more CH agents. Examples of some of the simple and more complex situations encountered to date in our laboratory and their tentative or predicted analyses are presented in the next section.

Tests for virulence can be conducted at any point in the analytical procedure but usually are not made until the degree of complexity of the situation has been estimated from SCI experiments, preliminary transmissibility experiments, and dsRNA analyses. Tests can be conducted using excised, dormant trunk sections of American chestnut trees with smooth bark and with living trees with smooth bark in the field (Elliston 1978). Excised stem tests are useful for detecting very weak to moderately weak pathogenicity and for estimating capacity to reproduce asexually. Field tests are preferred because they permit a much more thorough assessment of virulence. Three or four of the standard normal strains should be tested along with one or more test strains or, preferably, a test strain and one representative of each of the SCI types obtained from it. It is important that all strains in a test be inoculated into each tree because highly significant differences have been found in canker development in different trees but not within individual trees, so long as smooth bark is inoculated and the basal region of the tree is avoided (Elliston this proceedings; unpublished data). The Latin square experimental design is ideally suited for these experiments. Also, trees 10 cm or more in diameter at 1.4 m are preferred because tests then can be carried well into the second growing season. This is desirable because the hypovirulence of some strains is not very evident until the second growing season (Elliston unpublished data). Although Anagnostakis and Waggoner (1981) found rate of canker expansion during one growing season useful for comparing short term pathogenicities of strains and mixtures, the many observations required make this approach impractical for long term tests.

Ideally, inoculations should be superficial and made in May or early June, using inoculum taken from the advancing margins of actively growing, vigorous colonies. If inoculum is used from old cultures or from colonies in one of the transient abnormal states referred to earlier, infection often fails or canker development is retarded or otherwise abnormal (Elliston unpublished data). A minimum of five observation dates are recommended unless the test strain is markedly hypovirulent. The first observations should be made in late July or early August (canker area, degree of superficiality, and abundance of stromata and pycnidia), the second in late November or early December (canker area, degree of superficiality, abundance of stromata with perithecia), the third in late March or early April (canker area, degree of superficiality, abundance of stromata with perithecia), the fourth in late July or early August of the second growing season (canker area, degree of superficiality, abundance of new stromata with pycnidia), and the fifth in late November or early December of the second season (canker area, degree of superficiality, abundance of new stromata with perithecia). This set of observations should give an estimate of virulence for the test strain(s) and SCI. To determine the effects of these agents in strains typical of the species, similar tests would be conducted with sets of infected and uninfected standard strains.

If it is not clear from the virulence test that a strain is *E. parasitica*, this can be determined by a mating type test (Anagnostakis 1979).

Examples of Simple and Complex Hypovirulent Strains from Nature

Figure 2 illustrates the tentative analyses of three simple strains, EP-234, 418, and 60. Figure 3 is the predicted analysis of EP-419, and Figure 4 is a partial analysis of EP-405. Both EP-419 and EP-405 are complex strains. Strain EP-234, from an abnormal canker on *C. dentata* in Tennessee, contains one virus-like CH agent, H_{T2}, which severely weakens virulence. Strain EP-418, from an abnormal canker on *C. sativa* in southern Tuscany, Italy, contains one virus-like CH agent, H_{I2}, which prevents perithecium formation and stops canker development after the first growing season. Strain EP-60, from an abnormal canker on *C. dentata* in western Michigan, contains two virus-like CH agents: H_{M1}, which severely weakens virulence, and H_{M2}, which weakens virulence considerably but does not prevent formation of perithecia and normal ascospores. When present together in a strain, effects of H_{M1} are dominant. Strain EP-419, from the same canker as EP-418, appears to contain nuclei of two types, CH agent H_{I2}, and probably one or more other virus-like CH agents (Figure 3). Finally, strain EP-405, from an abnormal canker on *C. sativa* in central Tuscany, Italy, appears to contain nuclei of three types and one virus-like CH agent similar to H_{I2}. Other even more complex strains, e.g., EP-90 from Michigan, have been encountered, but they have not yet been analyzed.

The complex Italian CH strains, such as EP-419 and EP-405, are intriguing. Accumulated evidence suggests that they might arise from simple strains infected with H_{I2}, or other CH agents like it, by processes of 'degeneration'. Degeneration may result from nuclear mutation, to yield a less virulent heterokaryon, combined with mutation of the CH agent to yield a mixture of closely related CH agents. The cytoplasm of strains infected with certain CH agents may permit heterokaryons to persist. The degeneration of H_{I2}-infected strains to the H_{I1}-infected state, which has occurred occasionally

in the laboratory, may be due to these mutations. The hypothesis for this process of degeneration is depicted in Figure 5. A process such as this may be occurring in chestnut blight cankers in Italy and may be responsible for their gradual deactivation. If so, the H_{I_2}-type CH agents may be the key CH agents in the natural recovery process occurring in Italy.

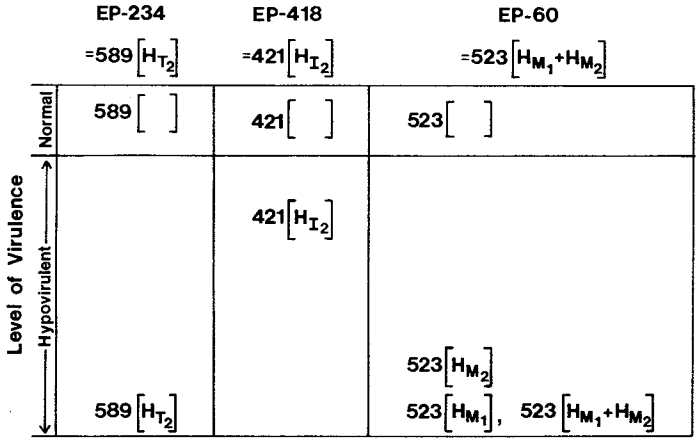


Figure 2. Tentative analyses of three simple CH strains of *Endothia parasitica*. Strain EP-234, from Bonair, Tennessee, is a normal strain, EP-589, containing one highly debilitating CH agent, H_{T_2}. Strain EP-418, from Mt. Amiata, Tuscany, Italy, is a normal strain, EP-421, containing one weakly debilitating CH agent, H_{I_2}. Strain EP-60, from Rockford, Michigan, is a normal strain, EP-523, containing two CH agents, H_{M_1}, which is highly debilitating, and H_{M_2}, which is moderately debilitating. The effects of H_{M_1} dominate when both agents are present.

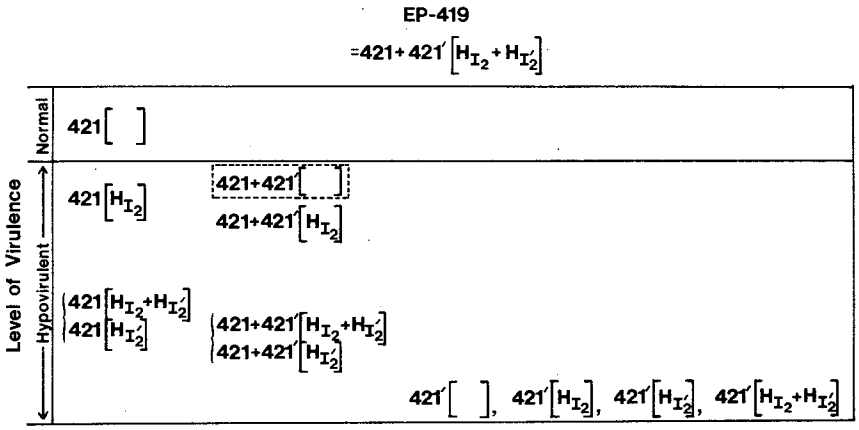


Figure 3. Predicted analysis of EP-419, a strain obtained from the same bark sample as EP-418 (see Figure 2), EP-419 probably is a complex strain containing normal type nuclei, represented by EP-421, abnormal nuclei, represented by EP-412[1], and two closely related CH agents, H_{I2} and $H_{I2'}$. Infection states preceded by a brace presumably have the same virulence level.

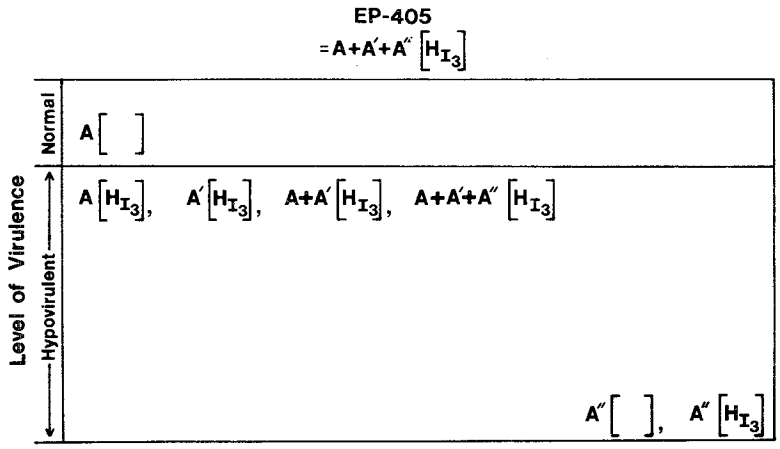


Figure 4. Partial analysis of strain EP-405 from Mt. Senario, Tuscany, Italy. It appears to be a complex strain containing normal nuclei, A, two abnormal types of nuclei, A' , and A'' , and one weakly debilitating CH agent, H_{I3}. Many probable infection states are not shown.

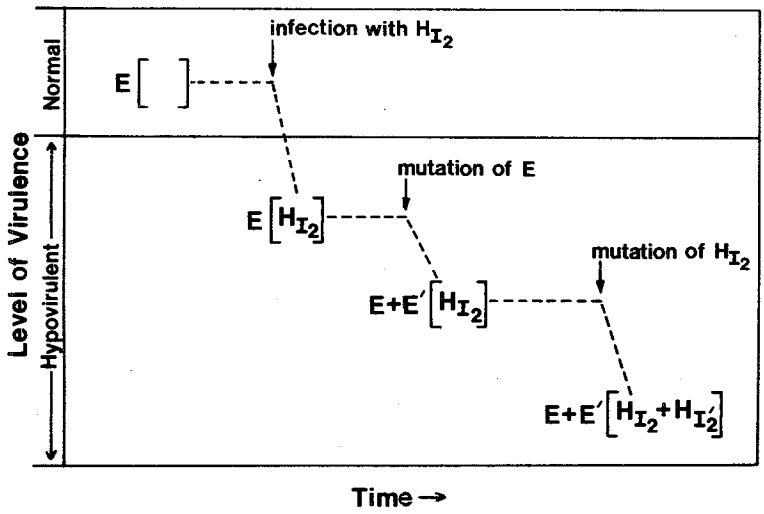


Figure 5. Hypotethical degeneration of a normal strain of *Endothia parasitica* after infection with CH agent H_{I2}.

Discussion

In *E. parasitica* growth and morphogenesis, i.e., development of form, and virulence are highly sensitive to changes in internal and external conditions. It is well known to anyone who has worked with this fungus that normal strains, i.e., those typical of the species, are quite sensitive to differences in growth medium, temperature, light intensity, and light periodicity. Perhaps we should be surprised that a strain of *E. parasitica* can be described as 'typical'. Yet, early investigators of chestnut blight marvelled at the high degree of uniformity among the thousands of cultures of this fungus that they examined (Shear et al. 1917).

These considerations, in addition to the high, rather uniform susceptibility of European and American chestnut to blight, probably account for the discovery of 'H' in this fungus. The first 'H' strains discovered in Europe and North America were isolated from abnormal cankers, have markedly low levels of virulence, highly abnormal cultural characteristics, and display high levels of curative capacity. Just because these strains and their unusual properties were discovered first is not a valid reason to confine the concept of hypovirulence in *E. parasitica* to them. The apparently continuous ranges of abnormalities found in the pathogenicity, reproductive capacity, and cultural characteristics of dsRNA-containing strains suggest that many agents may be involved. It appears also that abnormal nuclei might contribute to the reduced virulence of some strains.

The concept of hypovirulence suggested here accommodates all forms of subnormal virulence and classifies them according to cause. Such a concept provides a rationale for the detailed analysis of the causes of reduced virulence in individual strains. The procedures suggested for these analyses are tedious and time consuming, but they are necessary if we are to develop a fuller awareness and understanding of the hypovirulence phenomenon in *E. parasitica*.

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USDA FOREST SERVICE COOPERATIVE RESEARCH CHESTNUT PROGRAM 1978 TO 1982

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ABSTRACT.--*Cooperators in the USDA Forest Service American chestnut hypovirulent research program are identified. From 1978 to 1982, there have been 8 cooperators involving 15 studies. Approximately $400,000 have been obligated for this federal cooperative research effort.*

The impact of the loss of the American chestnut tree in this country is difficult to evaluate realistically (Figure 1). Of the more than 100 commercial Appalachian species, the American chestnut tree at one time comprised over 25 percent of the eastern hardwood forest. Research efforts to control the chestnut blight or drastically reduce the effect of the blight on American chestnut trees have not been successful. Recent developments in Europe have stimulated research efforts and renewed optimism toward controlling this disease. Certain less pathogenic (hypovirulent) strains of chestnut blight have been reported to inhibit growth of the virulent strains of the blight. Many European chestnut trees infected with the hypovirulent strains have recovered from the blight. Hypovirulence as a biological control has been the major thrust of new research.

In 1978, the USDA Forest Service, through the efforts of Senator Robert C. Byrd and his associates, provided funds for American chestnut research. To date, approximately $400,000 have been obligated for this research effort. Cooperators were selected to evaluate the hypovirulence phenomenon. The eight cooperators and major researchers are:

West Virginia University - Department of Plant Pathology
William MacDonald, Dale Hindal, and Walt Kaczmarczyk

Duke University - Department of Forest Pathology
William Stambaugh and Bruce Nash

University of Kentucky - Department of Plant Pathology
Louis Shain, Gerald Nordin, and John Russin

Virginia Polytechnic Institute and State University - Department of Plant Pathology
Gary Griffin, Fred Hebard, and John Weidhaas, Jr.

Utah State University - Department of Biology
Neal Van Alfen, James Bowman, and John Simmons

Concord College - Department of Physical Sciences
John Elkins and Bruce Given (West Virginia Department of Agriculture)

Southeastern Forest Experiment Station
George Kuhlman

Northeastern Forest Experiment Station
Alex Shigo

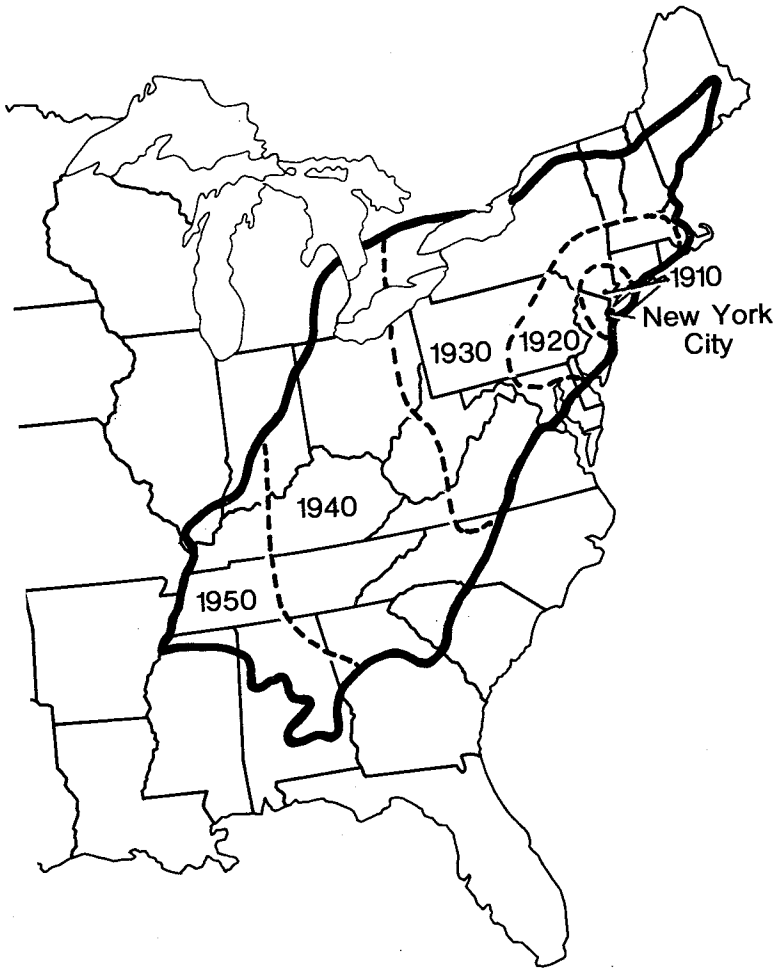


Figure 1. Spread of the chestnut blight in the eastern United States (Agricultural Research Service 1959). Wide solid line indicates natural range of the American chestnut, while dash lines indicate the periodic movement of the blight.

This cooperative research program involves a variety of research interests as indicated in the forthcoming papers and abstracts on hypovirulent and virulent forms of the chestnut blight. The major short-term objective of the USDA Forest Service cooperative research program is to evaluate the potential usefulness of hypovirulence as a biological control for *Endothia*

parasitica in the forest environment. At Duke University, research is being done on the transmission of hypovirulence in *E. parasitica* to chestnut and oak species in North Carolina. West Virginia University researchers are involved in a number of American chestnut tree field inoculation and blight dissemination studies. Also, vegetative compatibility, physiology, and molecular biology studies relating to *E. parasitica* are being done in the laboratory at West Virginia University. Canker development is being studied by George Kuhlman at the Southeastern Forest Experiment Station. Virulent and hypovirulent isolates are being evaluated as related to survival of American chestnut trees.

Hypovirulent and virulent strains of *E. parasitica* are being evaluated in "blight resistant and blight susceptible" chestnut trees by Virginia Polytechnic Institute and Concord College. Recent research data indicate that there may be some degree of blight resistance present in a large surviving American chestnut tree in Virginia. Also, histopathological studies are being done at Virginia Polytechnic Institute. The University of Kentucky scientists are evaluating the sporulation and dissemination of hypovirulent isolates of the chestnut blight with major emphasis on insects. At the Northeastern Forest Experiment Station, Alex Shigo is doing anatomical studies to evaluate and explain the American chestnut tree's defense system for the chestnut blight. John Elkins at Concord College has been evaluating the influence and utilization of chestnut tannins by *E. parasitica*. At Utah State University, researchers are evaluating how the hypovirulent factor reduces the virulence of the chestnut blight fungus. These researchers are assuming that hypovirulence of the blight is caused by double-stranded RNA that has been found in hypovirulent but not virulent isolates of the blight.

In addition to the above cooperators and their respective staffs, several non-funded cooperators are presently contributing or involved in the cooperative program. Researchers at the Connecticut Agricultural Experiment Station have made major contributions to the chestnut program. Several papers were given by the Connecticut staff in these proceedings. Also, participants from Michigan State University and Western Michigan University presented papers and have been consulted by many of the funded cooperators. The development of American chestnut trees in the Great Lakes Region, especially Michigan, is receiving considerable attention, and presently some chestnut trees in Michigan with superficial callousing cankers are apparently recovering from the blight.

During 1982, several additional studies will be funded. Research is progressing, and we must all keep abreast of the development in the various American chestnut research programs in this country. Eventually, researchers hope to solve the mystery of the chestnut blight and provide a means for the American chestnut to regain some of its usefulness in the eastern hardwood forest.

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Acknowledgment

The author appreciates and thanks James Lockyer, Northeastern Forest Experiment Station, Broomall, Pennsylvania, for completing Figure 1.

SUMMARY OF ENDOTHIA PARASITICA-HYPOVIRULENCE RESEARCH AT WEST VIRGINIA UNIVERSITY

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ABSTRACT.--*A background and overview of the chestnut blight-hypovirulence research program at West Virginia University is given. Field studies designed to deploy, establish and test the usefulness of hypovirulent strains as a biocontrol are described. Laboratory experimentation dealing with vegetative compatibility, physiology, cytology and molecular aspects of hypovirulence also are outlined.*

West Virginia University has had a long-standing chestnut research program. Until the 1970's this research was conducted within the Horticulture Unit as part of a larger project to find improved varieties of different nut-bearing species. Several investigators over the years devoted considerable time as part of this project to collecting and out-planting seedlings from surviving American chestnuts, but none of the chestnuts resulting from these efforts showed any significant degree of resistance. While the project generated considerable statewide interest, it suffered from meager financial support and the frustrating and overwhelmingly complex problems posed by chestnut blight.

The general interest in chestnut was rekindled at the WVU Experiment Station when Jean Grente's work on hypovirulence (Grente and Berthelay-Sauret 1978) was confirmed by researchers at the Connecticut Agricultural Experiment Station (Van Alfen et al. 1975). Awareness of the impact this phenomenon could have on the plight of the American chestnut resulted in several West Virginia legislative hearings to discuss the status of the species. Eventually, some state monies were appropriated to re-examine the entire problem. The interest shown by the West Virginia Legislature probably provided the driving force behind the current federal funding. In any event, the research efforts at WVU were reborn during the late 1970's. This led to the 1978 American Chestnut Symposium held in Morgantown, West Virginia that was sponsored jointly by the West Virginia University and the U.S. Forest Service. The symposium was attended by the majority of active chestnut researchers in the United States. However, the highlights of the meeting were papers given by Grente from France and Mittempergher and Turchetti from Italy (Grente and Berthelay-Sauret 1978; Mittempergher 1978; Turchetti 1978). Their presentations provided considerable insight into the phenomenon of hypovirulence and also served to brighten the outlook for American chestnut in the United States.

In summarizing hypovirulence research at West Virginia University, it is convenient to divide the discussion into field and laboratory studies. The field experimentation has been underway for a longer period of time and seems to be an appropriate place to begin.

Field Studies

Treatment Plots. The first attempts to utilize hypovirulent strains of *Endothia parasitica* were made in the late summer of 1976. Four study plots were established in a cut-over area of the George Washington National Forest where chestnut regeneration was abundant. Cankers on living chestnut trees were treated with a French-derived hypovirulent strain (EP-43) obtained from Richard Jaynes at the Connecticut Agricultural Experiment Station. Each canker was treated by introducing hypovirulent inoculum at two points on the lateral edges of the canker. The following spring, isolations were made from the treated cankers. The colonies that resulted were examined for morphologies similar to EP-43. Even though the majority of the isolates were morphologically similar to virulent isolates, a few distinctly white colonies, similar to EP-43 were recovered. Unfortunately the rate of successful canker control was extremely low.

Work by Sandra Anagnostakis on the relationship between vegetative compatibility (v-c) and the success or failure of canker treatment led to examination of the v-c types of virulent strains that were inciting cankers within the study area (Anagnostakis 1977). The eventual objective of this effort was to match each infection with vegetatively appropriate hypovirulent inoculum. Efforts to establish reasonably clear v-c groups followed, but required several months of laboratory testing. Upon returning to the study plots in the fall of 1977 to treat cankers with the appropriate hypovirulent inoculum, we were confronted with an overwhelming number of new infections that had developed during the season. It was apparent that the progress of the disease within our study plots was too rapid for such a methodical approach to canker treatment.

In the spring of 1978, a procedure of treating cankers with mixtures of hypovirulent inoculum was adopted following the report of the success achieved with this method by Jaynes and Elliston (1980) in Connecticut. Three mixtures were used that were comprised of isolates with similar cultural morphologies and often similar potentials to invade and sporulate in host tissues. A fourth mixture, "general", was also employed, that was composed of all hypovirulent strains contained in the other mixtures as well as several North American hypovirulent components. The mixtures used were as follows:

B-type. Isolates with white colony appearance and nonpathogenic,

JR. Isolates with orange-pigmented colony appearance and nonpathogenic,

Italian. Isolates slightly orange-pigmented and moderately pathogenic,

General. All of the above isolates and four North American hypovirulent strains.

From 1978 through the fall of 1981, these mixtures were used to treat new cankers as they arose in 24 study plots at three locations in West Virginia. During the 4-year period, almost 4,000 cankers have been treated on nearly 1,000 trees. A preliminary summary of this ongoing experiment is presented as an abstract by MacDonald (this proceedings).

Challenge Study. A study was established during the summer of 1978 to determine the role of vegetative compatibility to the success or failure of canker treatment. In this study, virulent cankers were established on healthy chestnuts using isolates of known vegetative compatibility type. The resulting cankers were then challenged with hypovirulent inoculum known to be vegetatively compatible or incompatible with the virulent isolate causing the canker. Individual hypovirulent isolates and the same hypovirulent mixtures described earlier were used to treat the artificially established infections. More virulent cankers were controlled than had been anticipated from their interaction in culture. Details of this study are presented in a paper authored by Double (this proceedings).

Scratch-Wound Study. During 1979, tests designed to establish reservoirs of hypovirulent inoculum on healthy chestnut stems were initiated in an effort to demonstrate natural dissemination of hypovirulent strains. To accomplish this wounds were made by scratching the bark at ground level, 1.5 m and 3.0 m off the ground and inoculum of different hypovirulent strains introduced into the scratches. Isolations have been made from all new infections that developed on the scratched trees. The cultural morphologies of these isolates have been examined and compared to that of isolates used as inoculum. The results of this experiment have provided the first evidence of the natural dissemination of hypovirulent strains on American chestnut following their artificial introduction. A detailed summary of these efforts are reported by Willey (this proceedings).

Periodic-Inoculation Study. Interest in determining the most appropriate periods to introduce hypovirulent inoculum led to a study of the effect of month of inoculation on subsequent canker growth and sporulation. This effort provided some unexpected findings with inoculations made in December and February. Even though inoculum remained viable within the inoculation site for over 2 years, cankers did not result. Double (this proceedings) summarized these results in an abstract.

Graduate Student Field Research. From 1978-80 field studies were undertaken by Richard Baird and Ronald Willey as part of their Master of Science degrees in Plant Pathology. Both students were interested in the growth and sporulation of a variety of hypovirulent and virulent strains. Willey used living chestnut stems for his evaluation whereas Baird examined saprophytic growth on red oak, red maple, and chestnut stems inoculated after they were killed by girdling. They found significant differences in the ability of virulent and hypovirulent strains to invade, sporulate, and survive in host and nonhost tissues. Even though the dead nonhost species did not support growth and sporulation as well as living chestnut, these abilities appear to be more dependent on the isolate tested than on the host. Complete details of their work are reported in their theses (Baird 1980; Willey 1980).

Summary. Field studies are particularly time and labor intensive. In some instances, field experiments have proceeded without the scientific background

information necessary to adequately plan them. Even though some work was obviously shortsighted, the ultimate goal to establish the usefulness of hypovirulence as a biological control has not changed. The efforts have raised a variety of questions that need to be addressed. Our field efforts have been conducted entirely in cut-over areas. These areas often were chosen out of necessity because they provide the abundant numbers of chestnut stems required for experimental replication. Even though a low incidence of chestnut blight has been a prerequisite for site selection, the number of subsequent infections builds so rapidly that it is often difficult to complete planned experiments. Treatment tests, designed to simply keep trees alive, have failed largely because of the large number of new infections that develop during a single season. In short, the virulent inoculum levels are so high that cut-over areas may present the most difficult circumstances for the introduction of hypovirulent inoculum. It may be more appropriate to look for trees that are relatively isolated and thus growing where inoculum buildup would be lower.

Time is the critical factor required for the transition of a virulent *E. parasitica* population to hypovirulent. This requirement places increasing importance on the information that can be gained by carefully observing and culturally examining new infections that develop over time in areas where hypovirulent inoculum has been introduced. Field studies often have dealt with the performance of hypovirulent strains and placed relatively little emphasis on following the course of new and old infections in introduction areas. This seems to be a particularly significant point now that evidence for the natural dissemination of artificially established hypovirulent strains exists.

Laboratory Studies

Vegetative Compatibility Tests. Over the past 5 years, we have attempted to determine the vegetative compatibility of *E. parasitica* isolates obtained from new infections that develop on the American chestnut within the eight treatment plots at Parsons, West Virginia. To accomplish this, *E. parasitica* isolates were obtained from canker margins prior to treatment and paired on an agar medium with test isolates from established vegetative compatibility groups (a group contains at least 2 isolates of the same compatibility). Of 880 isolates that have been paired, 795 (90 percent) have been placed in one of 37 different groups. The balance of the isolates tested are incompatible with the test isolates and each other. Even though a large number of vegetative compatibility types has been established, 47 percent of all the isolates fall within six vegetative compatibility types. Data obtained from this effort in combination with field treatment results will be analyzed to determine if a relationship exists between the vegetative compatibility type of the *E. parasitica* strain inciting a canker and the effectiveness of the hypovirulent treatment used.

Anastomosis and Cytology Studies. Hyphal anastomoses between vegetatively compatible and incompatible virulent and hypovirulent strains of *E. parasitica* was studied by Northup. Anastomoses of the hyphae-to-peg and peg-to-peg type were observed using light and scanning electron microscopy between vegetatively compatible and incompatible types. Collapsed cells were noted at the site of anastomosis. This work is described in a thesis by Northup (1981).

Efforts to view the cytology at the point of anastomosis will be continued by Newhouse with transmission electron microscopy. His initial observations of the cytology of virulent and hypovirulent isolates provided the first micrographs of virus-like particles in hyphal tip cells of *E. parasitica* strain EP-4. An abstract describing Newhouse's et al. findings appear in these proceedings.

Physiology Studies. Physiological studies are underway to determine if defined media will provide a distinct cultural separation of virulent and hypovirulent isolates. These efforts may also provide insight into the nutritional differences between virulent and hypovirulent isolates, as well as yield information on the physiological changes induced by hypovirulence. Progress of this work is reported in a paper by Hindal in these proceedings.

Molecular Biology of *Endothia parasitica*. A mycovirus from hypovirulent strain EP-43 was partially purified and characterized by Chmelo (1981). This work is presented in a paper by Chmelo and Kaczmarczyk (this proceedings).

In another abstract, Harper-Morris (this proceedings) reports her success in detecting RNA dependent-RNA polymerase in crude virus-like particle extracts of 14-day-old cultures of strain EP-43. Additional objectives of her work are to analyze the pH, template, and optimal temperature requirement, and optimal cofactor concentration of the enzyme.

Other studies, by Wheeler, are designed to examine the base-pair ratios of individual dsRNA bands formed in polyacrylamide gels and the possible presence of unusual nucleotides. He will examine the base-pair ratios by partial hydrolysis of the dsRNA followed by high performance liquid chromatographic separation. Search for unusual nucleotides will be attempted using thin-layer chromatography plates. Hopefully, this effort will lead to a rapid method for identifying various dsRNA species.

Summary. Many aspects of the basic biology of *E. parasitica* have not been studied for over 50 years. Now we are confronted with the phenomenon of hypovirulence which further complicates our appreciation of this fungus. The phenomenon of hypovirulence and biology of *E. parasitica* lend themselves to a variety of basic studies, but none currently seems more pressing than establishing the infectivity of the virus-like particles or the dsRNA associated with hypovirulent strains. The proof of infectivity should come with further refinements in procedure. Basic studies hopefully also will provide insight into the relatedness of the dsRNA associated with European and North American hypovirulent strains. Perhaps information on the dsRNA and its similarity to that found associated with other mycoviruses can also be learned. The most challenging task ahead, however, will be to coordinate basic studies with those designed to utilize this phenomenon as a biological control in the forest.

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SUMMARY OF RELATIONSHIPS AMONG SWOLLEN SUPERFICIAL CANKERS, SURVIVAL OF AMERICAN CHESTNUT TREES, AND HYPOVIRULENCE IN ENDOTHIA PARASITICA AT SOUTHEASTERN FOREST EXPERIMENT STATION

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ABSTRACT.--*Sixty-seven blight cankers on American and European chestnut trees yielded Endothia parasitica with hypovirulence (H) from 3 percent of the 1,240 isolates. One isolated American chestnut from near Bonair, Tennessee, yielded 28 of the 37 H isolates. American chestnut sprouts were killed 15 months after inoculation by virulent isolates but usually healed over after inoculations with H isolates. Bark patches from swollen superficial (SS) cankers used to inoculate American chestnuts have caused normal, lethal cankers 79 percent of the time after two growing seasons. Single compatible H isolates, combinations of H isolates including a compatible one, and 28 randomly selected H isolates when applied to wounded cankers have enhanced healing and prolonged tree survival in comparison to wounded check trees. Nonwounded cankers sprayed with conidia from compatible H isolates are healing as well as wounded cankers painted with mycelium in agar. Wounded and nonwounded check trees have 86 percent mortality in contrast to 29 percent tree mortality in the H treatments.*

Objectives

Three aspects of the research on hypovirulence being carried out at the Southeastern Forest Experiment Station are presented. The objectives were (1) to determine the frequency of occurrence of isolates of *Endothia parasitica* with and without hypovirulence (H) in swollen, superficial (SS) cankers ("healing cankers"); (2) to compare the effects of inoculation of American chestnut trees with virulent (V) and H isolates of *E. parasitica;* and (3) to increase survival of American chestnut trees by control of canker development with H isolates. An introduction, methods, results, and discussion sections are presented for each objective

Association of H Isolates with SS Cankers

Introduction

Subjective observations by European workers (Grente and Berthelay-Sauret 1978; Turchetti 1979; Turchetti and Marinelle 1979) form the basis for the hypotheses that natural intensification of H in *E. parasitica* is responsible

for increased survival of blight-infected European chestnut *Castanea sativa* in Italy and introduction of H isolates is providing biological control of chestnut blight in France. Survival of European chestnut in Italy is characterized by the presence of healthy bark callus beneath cankers according to Grente and Berthelay-Sauret (1978). If callus causes swelling and necrotic tissue is superficial, the canker is SS. Turchetti (1979) classified cankers as normal, intermediate, hypovirulent, or strongly hypovirulent. Normal cankers are depressed because the young xylem, cambium, and phloem are killed and these tissues collapse. Grente and Berthelay-Sauret (1978) state there was a direct relationship between the "degree to which the canker healed" and the number of white (presumably H) isolates of *E. parasitica* present. Recovery of white isolates varied from 5 to 100 percent. Completely healed cankers either yielded no isolates of *E. parasitica* or only white isolates from 10 to 20 percent of the samples. No details of sample size or sampling method were given. Many factors contribute to the disease cycle and therefore to disease expression. The disease pyramid consists of host, pathogen, environment, and time. Objective experiments should consider all four of these factors in relationship to any changes in disease expression.

Hypovirulent isolates of *E. parasitica* have been recovered from American chestnuts in the eastern United States (Day et al. 1977; Elliston et al. 1979; Jaynes and Elliston in press); however, the relative occurrence of H isolates and their association with SS and normal cankers have not been documented.

Methods

One study area near Buchanan, Virginia, was selected because trees with burrs were present, large SS cankers were abundant, and an H isolate was recovered from one canker in 1979. Trees near Robbinsville and Franklin, North Carolina, were sampled because they had swollen infections and at the latter site several trees were unusually large and had burrs. A tree at Bonair, Tennessee, was a source of H isolates in earlier reports (Elliston et al. 1979; Jaynes and Elliston in press). This tree was remote from other chestnuts. Two cankers on European chestnuts from Italy were sent by Turchetti in response to a request for superficial cankers to be used in grafting experiments; however, these cankers were normal in appearance.

At Buchanan, 42 cankers on 19 trees were sampled by removing 20 chips per canker. At Bonair, six cankers on one tree were sampled by removing 20 chips per canker. At Buchanan and Bonair, half the chips were from the canker margin and half were from the center of the canker. Eight SS cankers on five trees near Robbinsville were sampled by removing 25 chips from each canker. At Franklin, 10 SS cankers were sampled by removing 12 chips per canker. The two Italian cankers were sampled by removing 30 chips per canker. Chips were selected across the entire canker surface from the latter three sources.

Chips were surface-sterilized in 65 percent ethyl alcohol and plated on Difco potato dextrose agar (dPDA). Isolates of *E. parasitica* were subcultured on dPDA under white fluorescent lights to subjectively separate V and H isolates. Vegetative compatibility (v-c) groups were determined with the method of Anagnostakis (1977). Potential H isolates were paired with compatible V isolates on dPDA with methionine and biotin (dPDAmb) in a modification of Anagnostakis and Day's method (1977) to confirm the presence of a

cytoplasmic factor that was infectious and affected growth of V isolates. No cellophane is used and 5 mm agar discs with V and H isolates are placed 10 mm apart near the center of the plate. Plates are incubated at 24 C with a 16-hr-day (white flourescent light).

Results

Endothia parasitica grew from 85 percent of the sample chips (1,240:1,460) (Table 1). Subcultures were made of 910 *E. parasitica* isolates to study culture morphology, v-c groups, and capacity of suspected H isolates for conversion of V isolates. Thirty-seven isolates have been confirmed as having H present. The H isolates were common only in the Tennessee tree and were present in all six cankers. The canker margins yielded fewer H isolates (8:32) than did the canker centers (20:41). At Buchanan the four H isolates occurred in four different cankers. Three of these were SS and one was normal. One of the Italian cankers yielded five H isolates.

Table 1. Occurrence of hypovirulent (H) isolates of *Endothia parasitica* in normal (N) and swollen superficial (SS) cankers on American and European chestnut.

Location	Cankers (No.) N	Cankers (No.) SS	D.B.H. range of trees (cm)	Sample chips (No.)	*E. parasitica* Present (No.)	*E. parasitica* with H (No.)
Robbinsville, NC	0	8	3-8	200	187	0
Franklin, NC	0	10	5-30	258	176	0
Buchanan, VA	11	30	3-28	820	739	4
Bonair, TN	0	6	43	120	78	28
Italy	2	0	4-6	62	60	5
Total	13	54		1,460	1,240	37

Discussion

The intensive sampling of 65 cankers in the southern Appalachians indicates that H isolates are not commonly associated with SS cankers. In this region, environment, host response, or time (season of infection) may be causing the SS cankers.

At Buchanan, two of the four trees with H isolates died during 1981, whereas 17 of 37 with no H isolates present died. The tree at Bonair with 36 percent H isolates (28:78) appeared less vigorous in 1981 than it had in 1980. Since no trees with normal cankers were present in the Bonair area, no comparison of recovery from normal and SS cankers could be made. The presence of H isolates in one normal canker from Italy further confounds the association of H isolates with healing cankers.

The senescence syndrome (Jinks 1966) and H have many similar characteristics including a change in growth habit and transmission by cytoplasmic exchange. The senescence syndrome appears more frequently in old cultures of some fungi (Jinks 1966). This suggested that H isolates might be more common in cankers on trees that had been dead for several years. However, isolation of *E. parasitica* from 50 dead trees in Virginia and North Carolina did not yield H isolates. Nor did the frequency of H isolates in the Virginia trees with H in 1980 increase in 1981 after they died.

Inoculation of American Chestnut Trees with H Isolates

Introduction

Previously Kuhlman (1981) suggested white or H isolates were essentially nonpathogenic. Koch's postulates require a regular association of the pathogen with any given symptoms, recovery of the pathogen in pure culture, inoculation of the host, subsequent development of the same symptoms, and reisolation of the pathogen. Published results have confirmed only the occasional recovery of a debilitated pathogen, the H isolates.

The SS symptom may be the result of a balanced ratio of V and H mycelium in the outer bark. Initially I hoped to graft bark from SS cankers to healthy American chestnut stems to maintain this balance as well as the live host response in the cambium. However, the thick cambium and phloem from SS cankers did not match up with the thin bark on healthy trees, and the live host effect from the donor could not be maintained. Therefore, instead of grafts, inoculations were made.

The hypotheses for this group of experiments were: (1) SS cankers are the result of infection by H isolates of *E. parasitica*. (2) SS cankers are the result of a critical balance between V and H isolates which can be maintained in bark patches from SS cankers. (3) Colonization and sporulation by H isolates will be enhanced if the host substrate is weakened (a stem section rather than an intact tree).

Methods

Mycelium as inoculum. American chestnut saplings 1.3 to 2.5 cm in diameter and free of blight symptoms were inoculated by removing 7-mm bark discs with a cork borer and inserting dPDA discs of mycelium of V or H isolates. Inoculation points were covered with masking tape for 6 weeks. To facilitate colonization and sporulation by H isolates, the midpoint of 15-cm long segments of American chestnut stems (1.3 to 2.0 cm in diameter) were inoculated as above. In 1979 the experiment was installed at two locations (Buchanan, Virginia and Franklin, North Carolina), three inoculation dates (June to September at 6-week intervals at each location), and four V and four H isolates randomly selected from our collection for each location. In 1980 at Franklin two additional inoculation dates (April and June) and four Italian H isolates (selected as intermediately pathogenic by J. E. Elliston) were included with eight isolates used in 1979 at this location. Symptom development and sporulation was observed periodically through September 1981.

Bark patches as inoculum. In 1980 near Buchanan, 24 SS cankers were used as donors. Inoculations on nondiseased areas of 10 trees with SS cankers were

possible. These were called selfed inoculations. Bark patches from all 24 were put on healthy neighbors in a nearby stand, and on healthy trees in a stand near Black Mountain, North Carolina. Bark patches were also taken from the six SS cankers from near Bonair, Tennessee, and from the two normal Italian cankers and used for inoculations at Black Mountain. Bark patches from Virginia and Tennessee consisted of 2.5-cm squares, with one square (replication) from each canker inoculated at each location. Bark patches from the Italian cankers were disc extracted with a 9-mm diameter cork borer with 10 replications per canker.

In May 1981, 10 bark plugs were taken with an increment hammer (4-mm diameter) from each of the six SS cankers on the tree near Bonair, and from a large SS canker and two normal cankers on trees near Franklin, North Carolina. These plugs were used to inoculate healthy trees near Franklin. Treatments (bark plug source) were randomly assigned within each of 10 replications. A second study included bark plugs from nine SS cankers from Buchanan, and three SS cankers near Franklin. Bark plugs were collected with a 7-mm diameter cork borer. Treatments were randomly assigned within six replications. Canker development was observed in September 1981.

Results

Mycelium as inoculum. Chestnut sprouts were highly susceptible hosts for V isolates but were generally resistant to infection by H isolates regardless of season of inoculation (Table 2). September inoculations with V isolates

Table 2. Percentage of American chestnut sprouts on two sites with various symptoms in September 1981 after inoculations with virulent (V) and hypovirulent (H) isolates of *Endothia parasitica* on four dates

Symptoms	Month of inoculation							
	April[a]		June[b]		July[c]		Sept.[c]	
	V	H	V	H	V	H	V	H
Infected								
Dead	83	0	95	7	94	6	52	4
Alive	8	4	2	4	4	0	13	4
Swollen callus	4	8	0	17	0	8	0	0
Not infected								
Dead	0	0	4	8	2	17	6	19
Healed	4	88	0	64	0	69	29	73

[a] Percentages based on 24 samples (four isolates x six reps inoculated in 1980).

[b] Percentages based on 72 samples (four isolates x six reps x three sites, two in 1979, one in 1980).

[c] Percentages based on 48 samples (four isolates x six reps x two sites in 1979).

were less successful than those in April, June, or July. Sprouts were killed within 15 months after inoculation by V isolates.

H isolates from Italy selected for intermediate pathogenicity have produced some swollen callus and an orange color on 42 percent of the sprouts 13 to 15 months after inoculation. Further observations will be needed to determine if SS cankers result from this symptom. These isolates have produced normal symptoms on 21 percent of the sprouts and avirulent symptoms on 37 percent.

Colonization of segments of chestnut stem, as determined by sporulation, occurred readily following inoculations in June and July with V isolates but was infrequent following inoculations with H isolates or September inoculations with V isolates (Table 3). Similar stem-section inoculations near Franklin produced fewer infections.

Table 3. Colonization and sporulation by virulent and hypovirulent (H) isolates of *Endothia parasitica* on chestnut stem sections in April 1980 following inoculations on three dates in 1979 in Virginia

	Virulent			Hypovirulent		
	6/12	7/18	9/12	6/12	7/18	9/12
Colonized (percent of 24)	96	96	13	17	4	0
Length of sporulation (mm)	143	120	2	7	<1	0

Bark patches as inoculum. The 84 bark patches from Virginia, Tennessee, and Italy in 1980 have initiated lethal or girdling cankers on 79 percent (33 to 92 percent) of the inoculated trees (Table 4). Even bark patches put on nondiseased portions of the same tree (selfed) had 60 percent dead or girdled. Heavy callus with color, a possibly favorable symptom, is present on only 5 (0 to 33) percent of the inoculations. The 1981 inoculations were only 4 months old when these readings were made and are included to show the early trend toward low incidence of infection and high incidence of healing callus or no symptoms from SS sources contrasted with high infection (95 percent) from normal cankers.

Discussion

Neither mycelium nor bark patch inoculations have produced SS infections. Most H isolates have failed to produce symptoms of infection during the second year following inoculation. This generally avirulent condition explains in part the infrequent occurrence of H isolates in the southern Appalachians.

Bark patch inoculations have the possibility of maintaining a delicate balance between the amount of V and H mycelium in SS cankers. In 1980, single replications were used from 30 SS cankers from Virginia and Tennessee. The original intent was to place patches on the same tree, a neighbor tree, and a tree on a distant site. This design should differentiate among host and environmental effects on development of SS cankers provided that this symptom developed at least on the same tree (selfed). Our hypothesis was that if the host was responsible for SS cankers, these would develop only

Table 4. Symptom development in September 1981 on American chestnut trees in Virginia (VA) and North Carolina (NC) following inoculation with bark patches from swollen superficial (SS) and normal (N) cankers from NC, VA, Tennessee (TN), and Italy (IT)

Canker inoculation				Dead	Infected	Type of callus	
Source	Type	Site	Bark patches	or with sprouts	other symptoms	Heavy orange	Healing or no symptoms
			No.	Percent			
1980							
VA	SS	Selfed	10	60	0	20	20
VA	SS	Neighbors	24	92	8	0	0
VA	SS	NC	24	79	17	0	4
TN	SS	NC	6	33	17	33	17
IT	N	NC	20	85	5	0	10
1981							
TN	SS	NC	60	0	32	17	52
NC	SS	NC	28	4	29	25	43
VA	SS	NC	54	0	31	19	50
NC	N	NC	20	0	95	0	5

on the same tree, if local environment was responsible, SS cankers would develop on selfed and neighbors. If SS cankers developed on selfed, neighbors, and a distant host, then only the pathogen was responsible for the symptom. The limited number of trees available for selfing and the low frequency of H weakened this design. In 1981, the cankers with H isolates present were used as the major source of bark patches and 6 to 10 replications were used to increase the possibility for SS canker development. After 4 months, canker development is much less frequent from SS sources than from normal canker sources.

The Italian H isolates selected for intermediate pathogenicity seem to have potential for producing SS cankers since 42 percent of these inoculations have swollen callus and orange color after two growing seasons. However, less debilitated H isolates must not be needed for optimizing survival and spread in Europe because Grente, with years of field experience, furnished highly debilitated H isolates for the initial work in the United States (Day et al. 1977; Van Alfen et al. 1975). Furthermore, highly debilitated H isolates were recovered from SS cankers on American chestnut in this and other studies (Day et al. 1977; Elliston et al. 1979; Jaynes and Elliston in press), indicating that these isolates are capable of surviving under some circumstances.

Control of Canker Development with H isolates

Introduction

The goal of all research on hypovirulence in *E. parasitica* is to prolong the life of American chestnut trees. Reports from Connecticut have indicated

that treatment of cankers with H isolates slows canker development and reduces tree mortality after two growing seasons (Jaynes and Elliston 1978; Jaynes and Elliston 1980; Van Alfen et al. 1975). Studies were established in North Carolina and Virginia to follow tree survival for several years after H treatments.

Methods

In each of the four studies, cankers initiated by five V isolates were treated 1.5 months after inoculation with H isolates.

Study 1978-F. Near Franklin, North Carolina, in July 1978, half the cankers were wounded by cutting through the bark with an ax in several places prior to treatment of all cankers with an agar slurry (check) or an agar slurry of a culture of a compatible H isolate. Cankers on all nongirdled trees were wounded and treated again in May 1979.

Study 1979-F. Treatments were applied 1.5, 10, and 24 months after June 1979 inoculations near Franklin to ax wounds through the canker. Treatments included an agar slurry check, a compatible H slurry for each V isolate, the five compatible H isolates in a slurry, and a random selection of 28 H isolates in a slurry.

Studies 1979-Va. and 1980-F. Agar slurries were applied to wounded cankers and conidial suspensions or water to nonwounded cankers 1.5 and 10, and 24 months after inoculation near Buchanan in 1979 and 1.5 and 10 months after inoculations near Franklin in 1980.

Results

In every experiment where H isolates were used to treat young cankers initiated by V isolates, survival of the trees has been improved over the check treatment(s) (Tables 5 and 6). The oldest experiment, 1978-F, has gone through four growing seasons (37 months). In this experiment all check trees died on the average of 19 months after inoculation, whereas 45 percent (18:40) of the treated trees are still alive. Although the treatment has kept the trees alive, some cankers are still active. During the 1981 growing season, five trees died, two others have sprouts below the cankers, and seven have signs of the fungus.

In study 1979-F, wounded cankers treated with combinations of H isolates have improved tree survival. In studies 1979-Va. and 1980-F, trees with nonwounded cankers treated with conidia from H isolates have better survival than trees in the check treatments (Table 6).

Discussion

Trees with cankers survive longer if treated with H isolates than do the check treatments. The average percentage of trees dead in the six check treatments is 86 percent (Tables 5 and 6), whereas only 29 percent of the trees in eight H treatments have died. In the oldest experiment, 45 percent of trees with treated cankers are still alive after 37 months which is nearly double the survival time after inoculation for all trees with check cankers

Table 5. Effect of wounding and hypovirulent (H) isolates in agar treatments on symptom development after inoculation of American chestnut trees with virulent isolates of *E. parasitica*

Study	Duration	Trees per treatment	Symptoms	Treatment: Agar check	Compatible H in agar	5 H's in agar	28 H's in agar
	Months			Percent affected			
1978-F	37	40	Infected:				
			Dead	100	55	--	--
			Girdled	0	5	--	--
			Not girdled	0	25	--	--
			No symptoms	0	15	--	--
1979-F	25	25	Infected:				
			Dead or w/sprouts	60	28	28	16
			Not girdled	36	28	16	44
			Not infected:				
			Healed	0	28	48	24
			No symptoms	0	12	4	4
			Dead	4	4	4	8

Table 6. Comparison of wounded and nonwounded treatments before hypovirulence (H) treatments on survival of American chestnut following inoculations with virulent isolates of *E. parasitica*

Study	Duration	Trees per treatment	Symptoms	Treatment: Wounded, agar check	Non-wounded, water check	Wounded, H in agar	Non-wounded, H conidia
	Months			Percent affected			
1979-Va.	25	25	Infected:				
			Dead	92	92	27	12
			Not girdled	4	0	27	48
			Not infected:				
			Healed	4	4	31	28
			Dead	0	4	15	12
1980-F	15	40	Infected:				
			Dead or w/sprouts	73	88	3	15
			Not girdled	28	8	33	43
			Not infected:				
			Healed	0	5	65	35
			Dead	0	0	0	7

(Table 5). Slurries of a random selection of 28 H isolates were as effective in reducing tree death due to inoculations as were slurries of a compatible H or five H's including the compatible one. Most interestingly, conidial suspensions from compatible H isolates have increased tree survival even though the cankers were not wounded prior to treatment.

Enhancing survival of American chestnut trees seems to be an appropriate area for additional research. In the 1979-F experiment 28 randomly selected H isolates were used in one treatment. This number was selected because earlier results had suggested 25 to 34 percent of any confrontations between any virulent and any H isolate would result in conversion of the virulent isolate to H (Anagnostakis and Day 1979; Kuhlman 1978). To ensure conversion of 95 percent of the virulent isolates, a conservative 20 percent conversion rate required 14 H isolates which was then doubled.

Jaynes and Elliston (1978) have reported reduced canker growth four months after treatment with conidial suspensions but the reduction was less than that provided by agar slurries to wounds. Conidia were more effective on wounded cankers than on nonwounded cankers (Jaynes and Elliston 1978). The present study indicates that H isolates compatible with V isolates can retard canker development and increase tree survival for several seasons. If conidial suspensions from a random selection of H isolates can provide similar control of natural cankers, control may be feasible for orchards and high-value recreational areas.

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SUMMARY OF CHESTNUT RESEARCH AT DUKE UNIVERSITY 1980 TO 1981[1]

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ABSTRACT.--*Research on the potential for transmission of hypovirulence in Endothia parasitica to chestnut Castanea and oak Quercus in North Carolina is summarized and updated since the last U. S. Forest Service American chestnut cooperators' meeting held January 1980 at Pipestem, West Virginia. Plans for future directions in this research are discussed.*

By way of introduction and since two major studies in our program will be presented as separate papers, we will mention these first but only in terms of their status for the future. All other subprojects will follow by topic, summary of work accomplished, and future plans.

Incidence of *Endothia parasitica* in North Carolina

This 33-county survey of the mountain and Piedmont regions of the state is now complete, including vegetative compatibility (v-c) typing of 410 *E. parasitica* isolates thus derived, and will be reported later by Bruce Nash. This phase of our work is now terminated and no continuation along these lines is planned.

Detection and Evaluation of Survival in American Chestnut

Preliminary data from a four-tree sample, to date, on this project will be presented separately in these proceedings by this senior author.

Our plans, which are detailed in the paper, can now be implemented with the appointment of a research assistant this January 1982. The immediate winter schedule calls for: 1) mail survey to mill owners, buyers, and logging contractors in the mountain and foothill counties of North Carolina to enlist their help in locating large (21.6 cm d.b.h.) American chestnuts, *Castanea dentata*, for hypovirulence/resistance evaluation; 2) collection and storage of scionwood from all study trees for spring grafting attempts to Chinese chestnut, *C. mollissima*, rootstock; and 3) completion of isolate screening

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[1]Under title of, "Potential transmission of hypovirulence to hosts of *Endothia parasitica* in North Carolina," this research was partially supported by grants from the USDA, Forest Service and the H. Smith Richardson Charitable Trust.

from study trees to detect within-tree v-c patterns, abnormal cultures, and conversion ability of appropriate pairings. Next spring, pathogenicity trials with all suspect hypovirulent (H) isolates from the study trees, or a representative sample will be compared with virulent (V) isolates via inoculation of American chestnut sprout stems in Watauga County provided for our use by the Johnson Lumber Company of North Wilkesboro. The remainder of the field season will concentrate on intensive sampling of and isolate processing from additional study trees located from our mail survey and associated infected hosts found within 152 m of each study tree. Four candidate trees (d.b.h. range, 29.0 to 82.3 cm) have already been located for possible study selection in 1982.

Epidemiology of *E. parasitica* on the Duke Forest

Epidemiological monitoring of *E. parasitica* on the Duke Forest is underway and is focused upon a ± 50-year-old, 3.8 ha Asiatic chestnut *C. crenata* and *C. mollissima* plantation, representing an established epidemic, and two, ca 0.4 ha plantings of 2-0 Chinese chestnut stock planted in the spring of 1980 and 1981, representing potential for epidemic development. All three plantations have been stem mapped and canker incidence data is now being collected from the older one while new infections in the younger ones will be monitored by means of annual examination. Isolates will be obtained from all infected trees, all new infections, and from the older plantation, all cankers per tree in at least 10 percent of the multiple-cankered stems. All isolates will be tested for intra- and inter-tree v-c type frequency and distribution; culturally abnormal isolates will be processed for hypovirulence characteristics.

Beyond each plantation perimeter, all oak hosts *Quercus alba, Q. coccinea,* and *Q. stellata* to a distance of 305 m will be located and sampled for *E. parasitica* isolate yield and v-c/possible H-strain characterization.

A student project (Leininger 1981) which involved sketch mapping of and collection of incidence data from 220 native oaks in a 32 ha stand adjacent to the oldest Asiatic chestnut plantation, showed infection of white, post, and scarlet oak, on an isolation basis, at levels of 1.8, 21.4, and 37.5 percent, respectively. Isolates from three white, 18 post, and 24 scarlet oaks were grown in opposition culture, according to the methods of Anagnostakis (1978), with yields of six definite and seven possible v-c groups. Two v-c types, in particular, were consistent within group infections of five and 11 trees, primarily composed of scarlet oak.

More recently, all of these isolates have been grown in 9 cm potato dextrose agar (PDA) plates through three successive transfers; two from post oak and four from scarlet oak, all of which appeared normal initially, are now showing decided growth reduction and irregular colony margins. These will be tested further for conversion ability, using the methods of Anagnostakis and Day (1979), and pathogenicity, while the tree sources will be sampled intensively for additional isolate yield and characterization.

Cross-Inoculation Study

Ten randomly-chosen isolates of *E. parasitica* isolated from two sources each of American chestnut, Chinese chestnut, post, scarlet, and white oaks were

cross-inoculated on May 23 to 28, 1980, and replicated again on June 24 to 26, 1981, into e ch host represented. Each of the 10 isolates, plus a control, was replicated on at least 10 individuals of each host (total of 1100 inoculations). Inoculations of American chestnut were done at the Coweeta Hydrological Laboratory Forest on different dates as travel would permit.

To date, only the chestnut species inoculations have been sampled and rated, primarily to avoid canker coalescence, whereas the very slow canker development in the oaks has postponed canker evaluation until at least the 1982 field season. It is evident, however, that isolates derived from the oak species are just as virulent on the chestnut species as isolates from chestnut.

Applied Hypovirulence in *Endothia*-Infected Live Oak

This student project (Blair 1981) showed that 11 of 16 virulent isolates of *E. parasitica* from live oaks, *Quercus virginiana,* at Fort Monroe, Virginia were converted to hypovirulent forms when placed in opposition culture with a slurry of seven known hypovirulent strains. Three weeks after treatment (July 2, 1981) of portions of cankers on the isolate-source oaks with ten replicates each of a slurry and spray formulation of the seven hypovirulent strains, hypovirulent strains were reisolated from all four slurry-treated cankers sampled, one of which also yielded normal isolates, but only from two of four spray-treated cankers. The treated cankers will be examined in July 1982 for canker change, if measurable, and isolate yield from all treatments will be evaluated.

Blair (1981) noted that two of the isolates from live oak behaved in plate culture like H-strain isolates. Since then, we have grown 15 of the live oak isolates, including the two in question, in 9 cm PDA plates through four successive transfers and have found that the two isolates and one more show cultural abnormality typical of H-strain isolates. These isolates will be tested for pathogenicity in American chestnut and the tree sources will be scrutinized for possible symptom correlations along with sampling for additional isolate yield characterization.

Insect Vector Studies

Studies of ants in association with cankered and uninfected scarlet oak and Chinese chestnut on the Duke Forest are being continued by Tim Albaugh for his MF project requirements. Development of an agar medium for selective isolation of *E. parasitica* from suspect ants compared chestnut bark, tannic acid (10 and 50 ppm), and PDA. Tannic acid at 0.5 percent gave best results and utility, being exceeded only by PDA in growth response of the fungus (5.9 versus 4.3 mm/day). Only tannic acid, however, showed selectivity when the different media were inoculated with a contaminated conidial suspension of *E. parasitica* in dilution plate assay.

At various times during July to August and October 1981, especially as weather conditions varied, ants were collected from cankered and uninfected scarlet oak and Chinese chestnut trees and plated as 1 ml dilution washes on 0.5 percent tannic acid agar. To date, the fungus has been isolated in greatest frequency and inoculum yields from *Aphaenogaster lamellidens* up to 2 days

following rain. Only the collections from infected scarlet oak in June to July were positive; stem washes from all ant-source trees also yielded the fungus on tannic acid agar.

Presently, mode of transmission is being studied by caging ants infested with *E. parasitica* in fresh wounds on intact trees and excised, end-sealed stem sections of scarlet oak and Chinese chestnut. In addition, by exposing ants to H-strain inoculum, attempts will be made to obtain conversion of compatible V-strains on Chinese chestnut by field caging experiments. Screening of host-associated ant populations for quantification of vectoring potential will be continued.

Another MF project study by Susan O'Bryan involves the field exposure of artificially-inoculated V- and H-strain cankers in excised oak and chestnut stem sections for seasonal monitoring of insect attraction. Insect species collected from these cankers in sufficient numbers will be divided into separate lots for reference and identification, screening for isolate yield, and wound-caging on Chinese chestnut. Only the pilot-testing phase of this study has been developed to date.

Etiology of *Endothia gyrosa* on Branch-Pruned Willow Oak

In this MF project study by Greenberg (1981), an 86-tree sample of pruned willow oak, *Q. phellos*, in Durham, North Carolina showed canker/dieback incidence of 42 percent. On average, infections involved less than four branches per tree at stem origin heights less than 7.6 m and occurred most frequently on smaller trees (25 to 64 d.b.h.; range to 102 cm).

Inoculations with *E. gyrosa* on freshly-pruned willow oak branch stubs on August 13, 1980 averaged 6.6 cm penetration with 7 of 10 showing fruiting of the fungus by mid-December, thus confirming a new host record. No additional work is planned.

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SUMMARY RESEARCH
SPORULATION AND DISSEMINATION OF HYPOVIRULENT STRAINS OF THE CHESTNUT BLIGHT FUNGUS AT THE UNIVERSITY OF KENTUCKY

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ABSTRACT.--*Old chestnut blight cankers support abundant sporulation of both Ceratocystis microspora and C. eucastaneae, and were more attractive to insects than younger cankers where Ceratocystis is absent. Additional research indicates that Ceratocystis-laden chestnut bark can function in attracting insects to non-chestnut substrates. Ceratocystis perithecia were observed two months after their inoculation into established virulent (V) or hypovirulent (H) cankers. These results suggest that introduction of Ceratocystis species into H cankers may enhance insect dissemination of sparsely sporulating H strains. In vitro studies suggest that establishment of Ceratocystis in blight cankers is enhanced by the action of Endothia parasitica to modify inhibitory compounds in healthy bark and to produce metabolites which directly stimulate Ceratocystis. Both C. eucastaneae and, to a lesser degree, C. microspora inhibited growth and sporulation of E. parasitica on artificial media. Host range studies with excised dormant stems show that, of 23 species tested, development of selected V and H strains was supported by Acer rubrum, A. pennsylvanicum, Quercus velutina, Q. rubra, Betula lutea, and Castanea dentata. Dissemination of auxotrophic H strains has not been observed in study plots after 2 growing seasons.*

Introduction

During the early part of this century, the American chestnut *Castanea dentata* was virtually eliminated from the eastern forest by *Endothia parasitica*, causal agent of chestnut blight. The rapid, efficient spread of this introduced pathogen was accomplished primarily by dissemination of conidia and wind-blown ascospores (Heald et al. 1915). Hope for control of the chestnut blight lies in the use of curative hypovirulent strains of *E. parasitica* (Van Alfen et al. 1975). However, many hypovirulent isolates sporulate poorly or not at all, and little is known of their dissemination in nature (Day 1978). If these strains are to provide control of the chestnut blight under forest conditions, they too must be disseminated efficiently. Hypovirulence research at the University of Kentucky has centered on attempting to enhance the sporulation and dissemination of these curative isolates. Some of the materials, methods, and results obtained thus far are described in the following sections.

Status of Insects as Vectors of Hypovirulent Strains

The role of insects as vectors of phytopathogenic fungi has been well documented (Leach 1940). Early investigations into the spread of the chestnut blight fungus included a consideration of insects as potential vectors. Although a number of insect species were found to be closely associated with blight cankers (Craighead 1912; Anderson and Babcock 1913), interest in this area rapidly declined as none of these species was capable of inflicting wounds in healthy chestnut tissue. However, transmission of hypovirulent strains does not require wound inoculation but simply movement of hypovirulent inoculum between existing infections (Day 1978). Insects, then, could play important roles as vectors of hypovirulent strains.

Species in the family Fagaceae support a wide diversity of insects (Baker 1972). Although no intensive sampling of insects of American chestnut has been published, it may be presumed that the diversity of its insect fauna compares favorably with that of other species in Fagaceae (Opler 1978). In the summer of 1979, studies were begun to catalog the insect species which frequent American chestnut stems and blight cankers. Insects were sampled from different chestnut substrates: old blight cankers, which were characterized by much necrotic tissue; young blight cankers, without extensive necrosis; and healthy tissue. The sampling method involved affixing adhesive-coated fiberglass screening to the chestnut substrates. These traps were removed and replaced bi-weekly.

Results from these studies indicate that the insect fauna of American chestnut is mostly confined to two orders, Coleoptera and Diptera. Of the species captured, the majority were from families which spend all or part of their life cycles associated with healthy or decaying woody tissue. The major Coleopteran families include Eucnemidae, Scolytidae, and Bostrichidae, while Sciaridae, Phoridae, and Dolichopodidae were the major Dipteran families. Families such as these have been considered as suitable candidates for vectors of hypovirulent strains (Opler 1978). Old blight cankers proved to be more attractive to insects than either young cankers or healthy bark, both in species diversity and total number of insects captured (Figure 1). Some families seemed to show a sequential arrival pattern while others were present in equally high numbers throughout the summer, indicating an abundance of insects present on chestnut over the entire growing season. These results suggest that such an abundance and diversity of insects should provide numerous candidates for vectoring of hypovirulent strains.

Ceratocystis Species as Surrogate Fungal Attractants for Insect Dissemination of Hypovirulent Strains

Many species of *Ceratocystis* are closely associated with certain insect genera (Hunt 1956). Several of these associations are instrumental in the epidemiology of a number of tree diseases, e.g. oak wilt (Jewell 1956), Dutch elm disease (Gibbs 1978), and *Ceratocystis* canker of deciduous fruit trees (Moller and DeVay 1968). Recently, two species of *Ceratocystis*, *C. microspora* and *C. eucastaneae*, were observed on chestnut blight cankers (Davidson 1978; Davidson and Kuhlman 1978). These species are not pathogenic to chestnut, and little is known of their relationship to virulent or hypovirulent strains of *E. parasitica*. Further examination of chestnut sprouts and blight

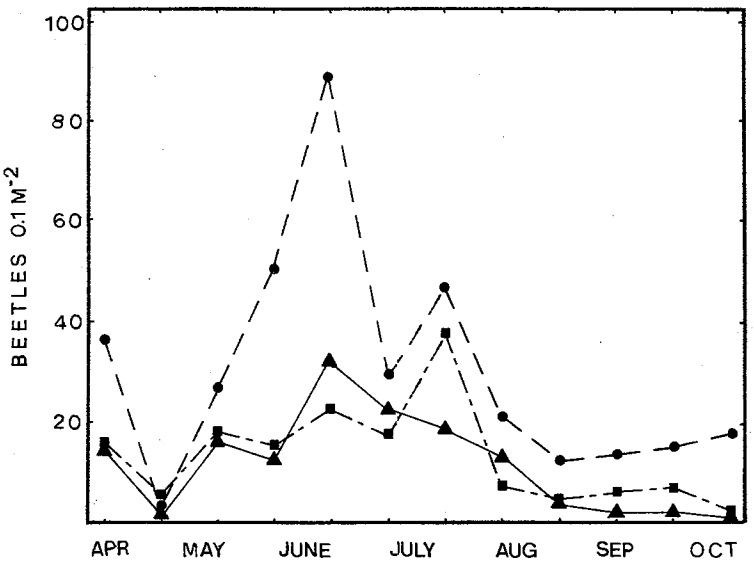


Figure 1. Total number of beetles trapped throughout the growing season from old blight cankers (● —— ●), young blight cankers (■— - —■), and healthy bark (▲——▲) of American chestnut.

cankers used for the insect surveys previously described showed perithecia of both *Ceratocystis* species present only on old, necrotic cankers. Thus, the presence of *Ceratocystis* may be partially responsible for the increased insect attractiveness of these old blight cankers.

To further test this hypothesis, field experiments were conducted during the summer of 1981 to determine if *Ceratocystis*-laden chestnut bark can attract chestnut insects to non-chestnut substrates. *Ceratocystis*-laden chestnut bark and healthy bark were fastened to sections of PVC plastic pipe which were anchored upright. Untreated pipe sections served as controls. Insects visiting these substrates were sampled according to methods previously described. Both insect diversity and total number of insects captured were consistently greater on *Ceratocystis*-laden chestnut bark than on healthy bark or untreated controls. These results, which are comparable to those obtained when entire stems were used as substrates (Figure 1), suggest that these *Ceratocystis* species are capable of attracting insects both to chestnut and non-chestnut substrates.

Due to the impaired sporulation of hypovirulent strains of *E. parasitica*, the successful introduction of these *Ceratocystis* species into hypovirulent cankers may prove useful in facilitating insect transmission of these strains. However, field observations have suggested that *Ceratocystis* perithecia commonly occur on old, necrotic cankers which sometimes no longer support *E. parasitica*. Therefore, work was initiated to determine if *Ceratocystis* could be established in younger cankers with abundant sporulation of *E. parasitica*. Stems of American chestnut were inoculated simultaneously with *C. microspora* and *C. eucastaneae*, singly and in combination, and with both virulent and hypovirulent strains. Both *Ceratocystis* species failed to develop in the ensuing blight cankers. However, when *C. microspora* and *C. eucastaneae* were

similarly inoculated into the centers of 13-month-old virulent and hypovirulent cankers, perithecia developed in inoculation sites and surrounding bark within two months.

The widespread dissemination of hypovirulent strains which has been reported in Italy (Mittempergher 1978) has yet to be repeated in the Unites States (Anagnostakis 1978; Jaynes and Elliston 1978). It may be significant that *C. microspora*, *C. eucastaneae*, and possibly several other *Ceratocystis* species have been observed on blight cankers of European chestnut in Italy (T. A. Turchetti personal communication). The attraction of insects to hypovirulent cankers colonized by *Ceratocystis* may provide needed assistance in the spread of hypovirulence in the eastern deciduous forest.

In Vitro Studies on Relationships Between *Endothia* and *Ceratocystis*

A series of experiments were conducted to further elucidate the relationships between *Ceratocystis* species and *E. parasitica*. A preliminary report of this work has been published (Russin and Shain 1981).

Ceratocystis microspora and *C. eucastaneae* were grown on Noble agar, a highly purified agar, containing aqueous extracts of blighted (BBE) or healthy (HBE) chestnut bark. Results are shown in Figure 2. With both species, growth and

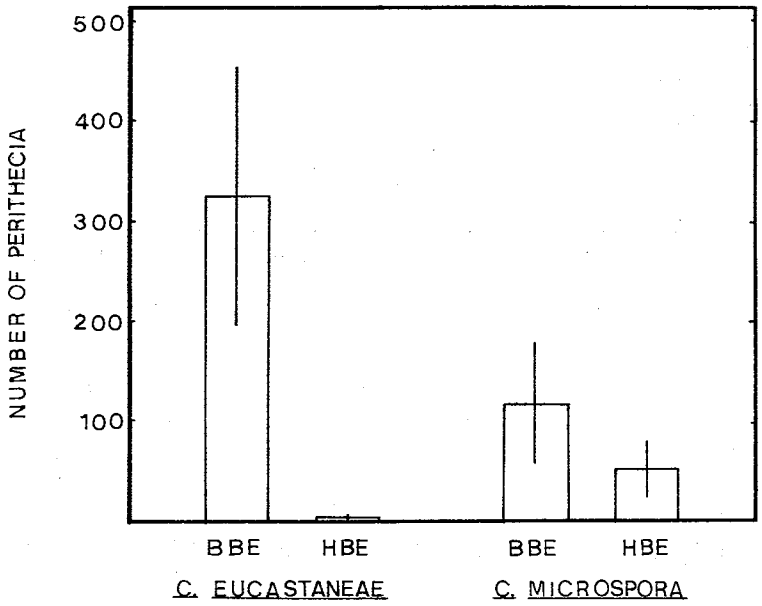


Figure 2. Perithecial production by *Ceratocystis eucastaneae* and *C. microspora* on media containing aqueous bark extracts of blighted (BBE) and healthy (HBE) bark of American chestnut.

perithecial production on BBE were significantly greater than those seen on HBE. Blighted bark, furthermore, contained significantly less condensed and hydrolyzable tannins than did healthy bark. Thin-layer chromatograms of

these aqueous bark extracts were subjected to bioassay with *Cladosporium cucumerinum*. With HBE, three zones were observed where sporulation of *C. cucumerinum* was inhibited, whereas no inhibition was seen with BBE or controls. These results suggest the presence of compounds, possibly tannins, in healthy chestnut bark which are inhibitory to growth and sporulation of both *Ceratocystis* species. To determine if modification of these compounds by *E. parasitica* would have a stimulatory effect on *Ceratocystis*, selected virulent and hypovirulent strains were grown over cellophane discs on HBE. After sufficient growth of *E. parasitica*, both mycelium and cellophane were removed from the medium surface and replaced with either species of *Ceratocystis*. Results are shown in Figure 3. All strains of *E. parasitica* used in these tests showed increased growth on HBE compared to NA controls. Growth of *C. eucastaneae* on HBE which had previously supported *E. parasitica* was increased over that on HBE alone. Perithecial production by *C. eucastaneae* was observed on BBE controls only. With *C. microspora*, both growth and perithecial production on similarly treated HBE was similar to that on untreated HBE controls.

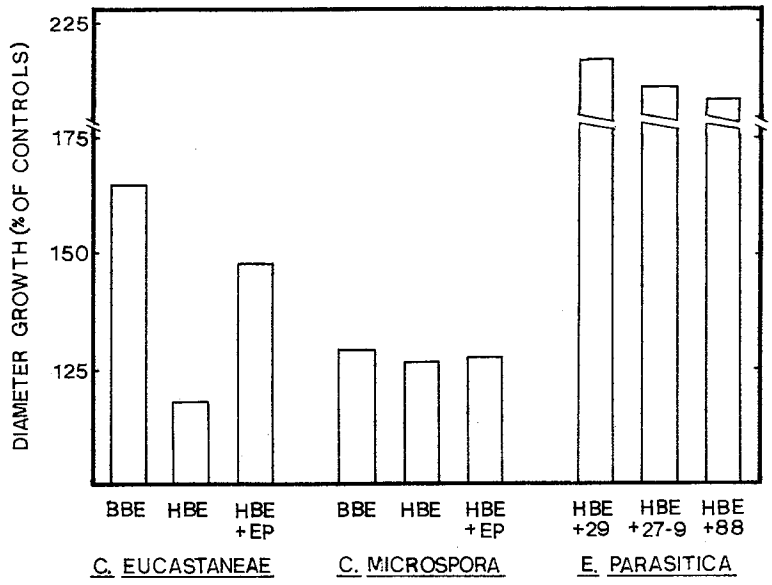


Figure 3. Diameter growth (percent of controls) of *Ceratocystis eucastaneae* and *C. microspora* on blighted bark media (BBE), healthy bark media (HBE), and healthy bark media which previously supported *Endothia parasitica* (HBE + EP). Values for *E. parasitica* represent diameter growth (percent of controls) of virulent (29) and hypovirulent (88, 27-9) strains.

Using the cellophane procedure, increases in growth of both *C. microspora* and *C. eucastaneae* were observed when these species were grown over Noble agar that had previously supported *E. parasitica* (Figure 4). When the order of these genera on cellophane-covered media was reversed, *C. eucastaneae* caused a large reduction in both diameter growth (ca. 60 percent) and concomitant pycnidial production by *E. parasitica*, whereas the effect of *C. microspora* was much reduced (Figure 5).

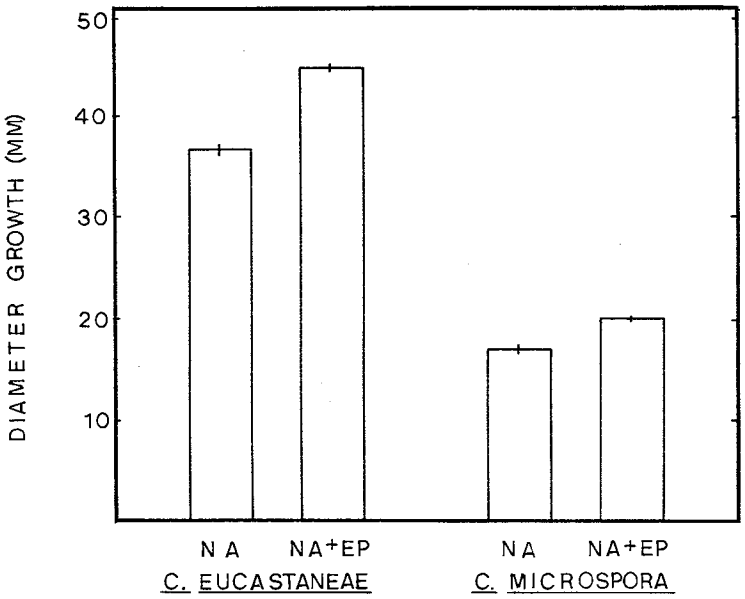


Figure 4. Diameter growth of *Ceratocystis eucastaneae* and *C. microspora* on Noble agar (NA) and Noble agar which had previously supported growth of *Endothia parasitica* (NA + EP).

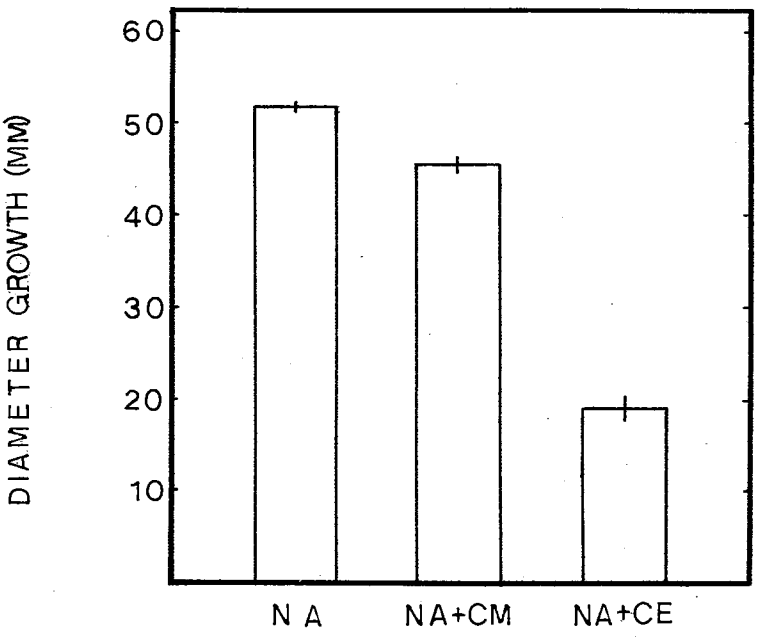


Figure 5. Diameter growth of *Endothia parasitica* on Noble agar (NA), and Noble agar which had previously supported growth of *Ceratocystis microspora* (NA + CM) or *C. eucastaneae* (NA + CE).

These results suggest a series of events which may be involved in colonization of blight cankers by *Ceratocystis* species. Establishment of *Ceratocystis* apparently is enhanced by a two-fold action of *E. parasitica*: modification of inhibitory compounds in healthy bark, and production of metabolites which directly stimulate *Ceratocystis*. That *C. microspora* is less affected by components of HBE than is *C. eucastaneae* may suggest a colonization sequence, with *C. microspora* becoming established earlier in blight canker development than *C. eucastaneae*. This is supported by greater inhibition of *E. parasitica* by *C. ecuastaneae*, as compared to *C. microspora*.

Host Range

Production of hypovirulent inoculum may be enhanced by establishment of these strains in woody species other than chestnut. Normal strains of *E. parasitica* have been reported to grow parasitically on white, post, and scarlet oaks (Nash 1981), and saprophytically on red maple, shagbark hickory, staghorn sumac, and several oak species (Anderson and Babcock 1913). Jaynes et al. (1976) tested 40 native and exotic woody species and found that only chestnut supported growth of selected virulent and hypovirulent strains.

Woody species which are frequently associated with American chestnut are being evaluated for efficacy in supporting sporulation of selected virulent and hypovirulent strains. To date, living trees and excised dormant stems of 23 species have been tested. Of species other than *C. dentata*, excised stems of *Acer rubrum*, *A. pennsylvanicum*, *Quercus velutina*, *Q. rubra* and *Betula lutea* have supported sporulation of virulent and hypovirulent isolates. These strains failed to become established in living stems in the field, suggesting possible roles for these species as saprophytic hosts. These woody species are also being screened for ability to support *C. microspora* and *C. eucastaneae*.

Field Dissemination

Upon clearcutting, sites which had previously supported chestnut regenerate to produce large numbers of chestnut sprouts. A number of study plots have been established in these areas to evaluate dissemination of hypovirulent strains.

Recovery of hypovirulent strains from study plots will be facilitated by the use of hypovirulent isolates bearing auxotrophic markers. These isolates were obtained from S. L. Anagnostakis or were prepared by pairing hypovirulent strains with methionine-requiring auxotrophs of normal strains in detached chestnut stems. Using a selective medium, dissemination of these isolates can be monitored under field conditions.

Naturally infected stems were removed from study plots to reduce levels of virulent inoculum prior to inoculation of the genetically marked isolate into centrally located stems. Subsequent reisolation from these stems confirmed the establishment of the marked strain. Surrounding chestnut sprouts were not wounded or wounded artificially during different seasons of the year.

Our limited results indicate that spread of marked strains has not occurred after 2 growing seasons. Although numerous new infections have arisen in

these plots, isolations from these cankers have not yielded the auxotrophic strains.

Plans for Future Research

Dissemination plots already established will be continually monitored for evidence of spread of the tagged strains. Additional plots also are being established to determine if association of *Ceratocystis* with the auxotrophic inoculum can enhance spread of these hypovirulent isolates within the study areas. Ongoing research will continue to examine additional woody species as possible hosts for hypovirulent strains of *E. parasitica*. Furthermore, excised dead stems also will be utilized to determine if these strains can serve as sources of inoculum for dissemination. Future plans for research with *Ceratocystis* include further investigations into the interrelationships between these species and *E. parasitica*. In addition, we will attempt to determine if a temporal arrival sequence exists for *Ceratocystis* and, if so, how that can be used to facilitate sporulation of these species and, ultimately, dissemination of hypovirulent strains. Hopefully, these efforts will yield information on how dissemination of hypovirulence can best be enhanced in the eastern forest.

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SUMMARY RESEARCH ON BIOLOGY OF HYPOVIRULENT AND VIRULENT ENDOTHIA PARASITICA ON BLIGHT-RESISTANT AND BLIGHT-SUSCEPTIBLE CHESTNUT TREES AT VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY

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ABSTRACT.--*Evidence is presented that some degree of blight resistance may be present in a large, surviving American chestnut tree. The evidence includes results of inoculations of stems excised from the tree, of grafted scions of the tree, and of the intact tree, including microscopical observations of such cankers. Additionally, the lengths of naturally caused cankers on the tree were measured over a 3-year period and seedlings of other large, surviving American chestnuts were inoculated. Results also are outlined of histopathological examination of the development of cankers incited by a virulent (V) and a hypovirulent (H) strain of Endothia parasitica on blight-resistant Chinese chestnut and blight-susceptible American chestnut, as are studies of epidemics of V strains of E. parasitica on American chestnut and an experiment on biocontrol by H strains of cankers caused by V strains.*

Introduction

The purpose of the studies detailed in this paper was to determine the cause(s) of survival of a large, American chestnut tree. We measured its blight resistance and the pathogenicity of the strains of *Endothia parasitica* which were attacking it. We hoped to assess the effect of blight resistance on disease control with hypovirulent *E. parasitica;* part of that assessment was a test of the potential of four hypovirulent isolates to biocontrol cankers of four virulent isolates on American chestnut stump sprouts (blight-susceptible). To help our understanding of hypovirulence and blight resistance, we also examined the time-course of canker development of a virulent (V) and hypovirulent (H) isolate of *E. parasitica* on the large, surviving American chestnut, on blight-resistant Chinese chestnut, and on blight-susceptible stump sprouts of American chestnut. Finally, to provide a foundation for understanding spread of hypovirulence, we examined the dynamics of the increase in blight incidence which occurs after clearcutting of

forested areas. Studies of the large, surviving American chestnut tree, the Fldg tree, are reported in detail in this paper and the results of biocontrol, histopathological, and epidemiological studies are summarized.

Methods and Results

Blight resistance in large, surviving American chestnut trees

The Fldg tree measured 39 cm in diameter at breast height (d.b.h.) in 1977, and 85 percent of its crown was alive. The presumed parent of the Fldg tree was located about 10 yards from it; the parent measured over 100 cm d.b.h. without its bark. Patches of bark were clinging to the presumed parent indicating that it had died within the last 10 years (Gravatt and Gill 1930). Another nearby large presumed seedling of the parent tree, the seedling being called the Flds tree, measured 18 cm d.b.h. with 20 percent of its crown alive in 1977. The Flds tree was killed by blight in 1980. Another nearby apparent seedling is approximately 15 cm d.b.h. Three to five other small American chestnut plants also are located near the Fldg tree.

The main bole of the Fldg tree is encased by a blight canker for 2.2 m above ground level. Isolated natural cankers also exist on the Fldg tree. Three natural cankers were treated on June 21, 1978, with the H isolate, EP-66 (Elliston 1978). Inoculations with EP-66 were made every 10 cm around the uninfected periphery of each canker. Three untreated natural cankers served as controls. The treated cankers increased by 8.8 cm in mean length between July 3, 1978, and March 8, 1981, and the untreated cankers increased 13.0 cm in mean length. There was no evidence of callusing on any canker.

In 1980, ten isolates were collected from the main bole of the Fldg tree and two from one of the isolated natural cankers treated with EP-66. The isolates were tested for pathogenicity (Griffin et al. 1978). Two of the isolates from the main bole were hypovirulent, in that the five replicate cankers of each isolate had a mean total length of less than 3.2 cm 4 months after inoculation. The cankers of the H isolates also were superficial in that they did not reach the vascular cambium except around the inoculation point. The cankers of each of the other V isolates had a mean total length exceeding 12 cm 4 months after inoculation. The cankers of the V isolates extended to the vascular cambium over most of their length and produced abundant stromata. Ten or more isolates also were collected from 12 additional large (mean d.b.h. over 50 cm), surviving American chestnut trees in Virginia and West Virginia. From most trees, about 20 percent of the isolates were hypovirulent. However, there were no hypovirulent isolates in 28 isolates from one tree.

Before giving further results of inoculations with the Fldg and Flds trees, it will be helpful to outline the kinetics of canker growth following artificial inoculation. Figure 1 shows the lengths of three individual blight cankers versus days after inoculation. The top two curves in Figure 1 are typical of most of the 24 cankers of this experiment. The bottom curve is the most atypical canker growth curve. In the typical canker growth curve, there is an initial rapid spurt of growth followed by a plateau of no increase in canker length. The plateau started about day 9 in this particular experiment. At day 26, a phase of linear canker expansion began and persisted until the tree was girdled by the canker. At this time, canker elongation often ceased, especially above the canker. Sometimes a short

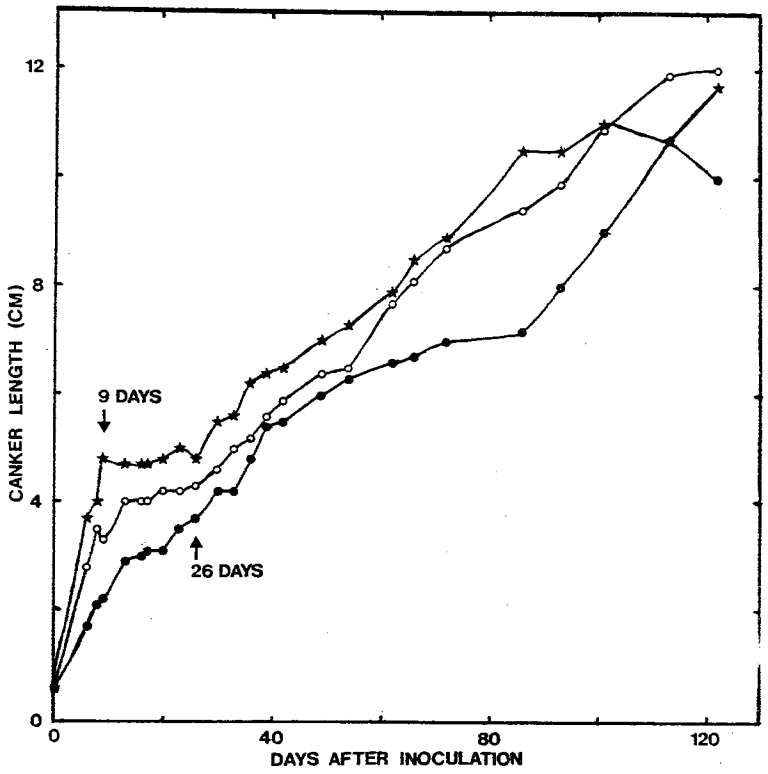


Figure 1. Lengths of three individual cankers on American chestnut seedlings from 6 to 122 days after inoculation with a virulent isolate, CR, of *Endothia parasitica*. Inoculations made June 7, 1979. The bottommost curve was from the most atypical of the 24 cankers. The top two curves are typical.

spurt of extremely rapid growth occurred at the time of canker girdling.

We collected scionwood material from the Fldg and Flds trees. Scions were collected from six other large (mean d.b.h. of 70 cm), surviving American chestnut trees and from two small, blight-susceptible American chestnut seedlings. In addition, seeds were collected from six large (mean d.b.h., 44 cm), surviving trees and three susceptible-type trees. Unfortunately, seeds from the Fld trees were unavailable. The scions were top-worked onto 15- to 30-year-old Chinese chestnut by bark grafting (Elkins et al. 1980). After germination and growth in 13 cm pots in the greenhouse, the seedlings were transplanted to a nursery plot, in rows 90 cm apart with 45 cm between plants in a row. Three years later, in June, 1980, each scion or seedling was inoculated twice to the vascular cambium with agar disks (1.5 mm diameter) of the V isolate of *E. parasitica*, CR. The cankers were measured every 3 to 4 days.

The inoculations of these plants (which included the H isolate, EP-66) were designed to detect blight resistance in them and to assess the effect of blight resistance on biocontrol of the V isolate by the H isolate. Unfortunately, except for two inoculations, the H isolate behaved as a virulent throughout the course of the experiment. Therefore, only the results for the V inoculations on grafted scions are presented here. It is suggested that the use of mycelial ball inoculum (Bazzigher and Schmid 1962) for the

small diameter inoculations would alleviate this complication, since older cultures, possibly with a higher dsRNA titer, could be used. In addition, many of the V inoculations failed on three of the seed sources, probably because the agar plates were allowed to become overheated. It is suggested that more careful attention to the condition of the inoculum might have eliminated the inconsistent results observed on these three seed sources.

Table 1 shows the mean diameter of the grafts at the inoculation point, canker lengths at 10 and 45 days after inoculation and the time of start of linear

Table 1. Canker growth statistics on grafted scions of large, surviving and small American chestnut trees inoculated with the virulent isolate of *Endothia parasitica*, CR

Scion Name [a]	Inoculations	Tree size	Stem diameter inoculation point [b]	Canker length [b] 15 days	Canker length [b] 45 days	Start of linear canker growth [b]
	no.		mm	mm	mm	days
Swent	2	large	4.1b	10.0bc	*	10.0c
GladA	10	small	22.1ab	18.7a	49.5a	12.8c
McDII	8	large	18.1ab	19.8a	49.5a	12.5c
GladB	4	small	10.9b	11.3bc	40.5ab	16.8bc
McDI	8	large	13.7b	18.9a	40.8ab	16.3c
Amhst	10	large	15.3b	12.0b	37.7b	13.2c
Weekly	10	large	15.1b	13.1b	36.9b	16.1c
Fldg	6	large	15.2b	11.0bc	32.0b	23.2bc
Flds	2	large	34.9b	18.0a	31.5b	33.5b
Gaul2	4	large	16.0ab	4.4c	10.3c	78.0a

[a] The start of linear canker growth was determined as the day after inoculation subsequent to which the increase in canker length from the previous observation exceeded 0.2 mm. Observations were 2 to 3 days apart, starting 5 days after inoculation. If one or two increases prior to day 25 were followed by three or more contiguous intervals where growth did not exceed 0.2 mm, the time of start was taken at first increase after that of more than 0.2 mm.

[b] Means within columns followed by the same letter are not significantly ($p < 0.05$) different by Duncan's multiple range test. There were two inoculations per scion.

* This graft was girdled and killed prior to 45 days after inoculation.

canker growth. The mean canker length at 45 days for the Fldg scions was significantly ($p<0.05$) shorter by Dunnett's test than the pooled mean for the two control scions (GladA and GladB) as was the mean time of start of linear canker growth in the Flds scions. However, Duncan's multiple range test revealed no significant ($p<0.05$) differences between the Fld scions and the GladB scions for these statistics. The small sample sizes may have contributed to the lack of sensitivity of the test. This is borne out by the results for the successfully inoculated seedlings (Table 2); there, many of the

Table 2. Canker growth statistics on seedlings of large, surviving blight-susceptible American chestnut trees inoculated with the virulent isolate of *Endothia parasitica*, CR

Seedling[b]	Stem diameter inoculation point[c]	Canker length[b] 15 days	Canker length[b] 45 days	Start of linear canker growth[b]
	mm	mm	mm	days
Wisc (su)	18.0bc	28.6a	64.2a	21.9b
McDI (sr)	25.3a	26.4ab	59.4ab	19.4b
McDII(sr)	17.5bc	21.4b	49.8bc	21.9b
Horn3(sr)	15.9cd	21.8b	48.7bc	25.2b
Gaul2(sr)	21.3ab	22.7b	47.7c	24.6b
Pea3 (sr)	11.6d	11.0c	25.7d	35.3a

[a] The start of linear canker growth was determined as the day after inoculation subsequent to which the increase in canker length from the previous observation exceeded 0.2 mm. Observations were 2 to 3 days apart, starting 15 days after inoculation. If one or two increases prior to day 25 were followed by three or more contiguous intervals where growth did not exceed 0.2 mm, the time of start was taken as the first increase after that of more than 0.2 mm.

[b] Seedling name, surviving (sr) or susceptible (su).

[c] Means within columns followed by the same letter are not significantly ($p < 0.05$) different by Duncan's multiple range test. There were five seedlings of each type with two inoculations per seedling.

seedlings from large, surviving trees gave canker growth statistics significantly ($p<0.05$) different from those for the seedlings of the susceptible-type trees (Wisc), even though the magnitude of the differences were not as great as was observed on the grafts.

We have performed additional experiments with the Fldg tree. We excised stems from it and inoculated and incubated them in the laboratory using the method of Elliston (1978). Table 3 shows the results of this experiment. The cankers on the Fldg tree were significantly ($p<0.05$) shorter than the cankers on the control tree and the WA tree, another large, surviving American chestnut.

We also inoculated the Fldg tree *in situ*. Using the EP-66-CR H-V pair, five H+V, V-alone, and H-alone, inoculations were made on 2 cm diameter branches on the Fldg tree, one inoculation per branch. The inoculations were made on June 21, 1978. On November 11, 1978, the V cankers had a mean and standard deviation of 5.1 ± 1.95 cm, the H cankers 2.8 ± 0.54 cm, and the H+V cankers 6.8 ± 1.41 cm. By October, 1979, two of the branches above the five V cankers were dead and three of the branches above the five H+V cankers were dead. The two lethal V cankers had measured 17 and 22 cm long and the

Table 3. Mean canker lengths on excised stems (branches) of two large, surviving and one small American chestnut tree inoculated with virulent isolates of *Endothia parasitica*, CR and Weekly

Tree[a/]	Tree size	Inoculations	Total canker length[c/]		Net canker[b/]	
			WK	CR	WK	CR
		no.	cm			
ST	Small	6	11.5a	11.8a	9.4a	9.5a
WA	Large surviving	6	8.8b	8.3b	6.6b	6.5b
FG	Large surviving	8	5.8b	6.2b	3.5c	4.2b

a/ Stump sprouts collected from a mature forest area in the Jefferson National Forest, Virginia, on February 21, 1979. Branches from large, surviving trees were collected on March 10, 1979.

b/ Means determined by subtracting initial lesion size, at 16 days, from total lesion size at 37 days at 27 to 28 C.

c/ Means within columns followed by the same letter are not significantly ($p < 0.05$) different by Duncan's multiple range test.

three lethal H+V cankers had measured 27, 30, and 17.5 cm long on June 18, 1979, one year after inoculation. Each of the three non-lethal V cankers were less than 2.5 cm long and the two non-lethal H+V cankers were 8.0 and 2.5 cm long on June 18, 1979. The non-lethal cankers did not grow further through October 31, 1980; most of them had ceased sporulating then, and some were hard to detect. The five cankers incited by the H isolate remained 6 cm long or less, and none of them killed the branches they were on.

Finally, we measured the lengths of cankers which had been made in connection with histopathological studies of canker development on the Fldg tree, on blight susceptible stump sprouts of American chestnut, and on blight-resistant grafted scions of Chinese chestnut, cultivar Nanking. Figure 2 shows the sum of the canker dimensions on the three hosts for the V and H isolates, CR and EP-66, versus days after inoculation in two experiments started July 4, 1978, and June 7, 1979. Each point in Figure 2 is a measurement of a separate canker. It can be seen that the cankers on the Fldg tree (denoted MR in the figure) were approximately the same length as, or shorter than, the cankers on the blight-resistant Chinese chestnut trees (denoted HR in the figure), and that, as expected, the cankers on the Chinese chestnut were shorter than the cankers on the American chestnut stump sprouts.

The canker length data indicate that resistance is playing a role in the survival of the Fldg tree. The findings that cankers on the grafts and seedlings of the G2 tree grew significantly ($p<0.05$) more slowly than cankers on all control trees indicate that there is heritable resistance to blight in

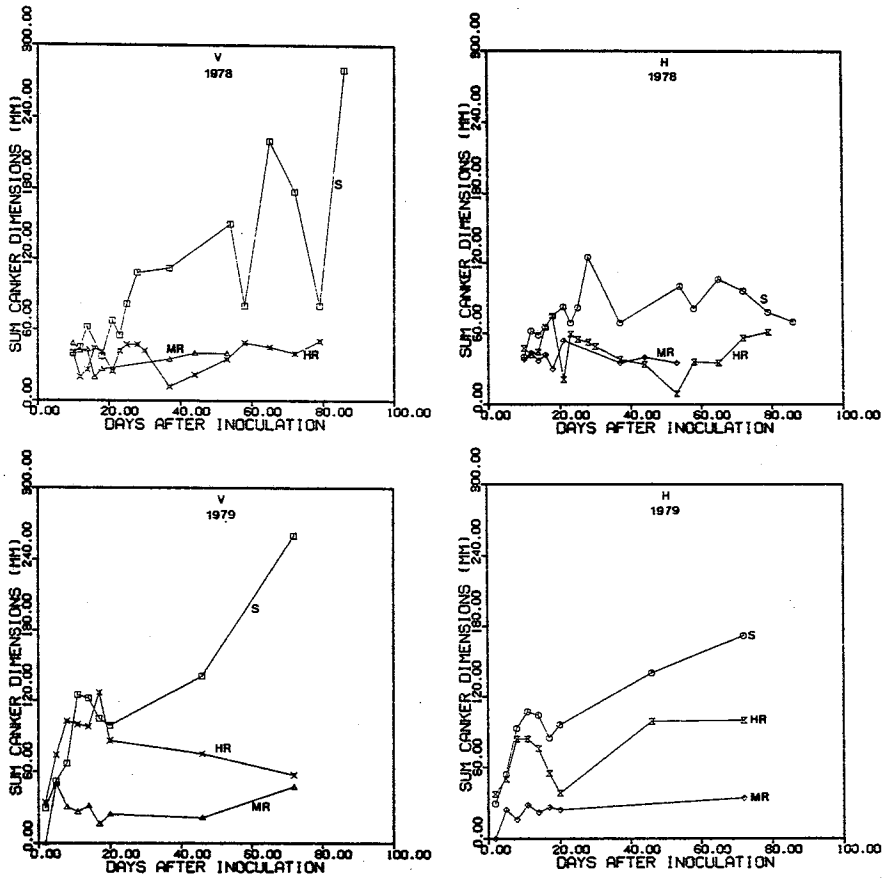


Figure 2. Sum of outer (at the outer periderm) and inner (at the vascular cambium) canker lengths and widths versus days after inoculatiion on July 4, 1978 and June 7, 1979, respectively. Of blight-susceptible (S) American chestnut stump sprouts, a moderately blight-resistant (MR), large, surviving American chestnut (the Fldg tree), and highly blight-resistant (HR) Chinese chestnut, cultivar Nanking, with virulent (V) and hypovirulent (H) strains of *Endothia parasitica*. The scatter in the data occurred because each point represents a separate canker. When there were duplicate cankers on one day for the S tree, the one with the shortest dimension is depicted.

American chestnut. The similar response of the grafts of the Flds and Fldg trees, which apparently are siblings, suggests that these plants also contain heritable blight resistance. Unfortunately, we have not been able to estimate the degree of heritability of blight resistance, because the cankers on the seedlings grew more rapidly than those on the grafts. As stated, about 20 percent of ten or more isolates each of 13 large, surviving American chestnut trees was hypovirulent. This, in conjunction with the above results, suggests that survival may be an interaction of resistance and hypovirulence. We do not know which is more important.

Histopathology

The scatter in the data points for Figure 2 (also for Figures 5 and 6) occurred because each point is a measurement of a separate canker. Each canker was processed for light microscopy to study canker development.

Comparing the curves in Figures 1 and 2, it can be seen that they have the same general outline for the blight-susceptible American chestnut stump sprouts, namely, that there is a rapid early flush of growth followed by a plateau before linear canker expansion begins. The rapid phase of growth was characterized by individual hyphae growing intra- and intercellularly (Figure 3a). The formation of a zone of lignified cells around the point of inoculation (Figure 3b) appeared to halt the expansion of these hyphae. The plateau phase of canker development then began until, in susceptible trees, a mycelial fan formed (Figure 4a) and expanded through and beyond the lignified zone. Linear canker growth was accompanied by the expansion of mycelial fans.

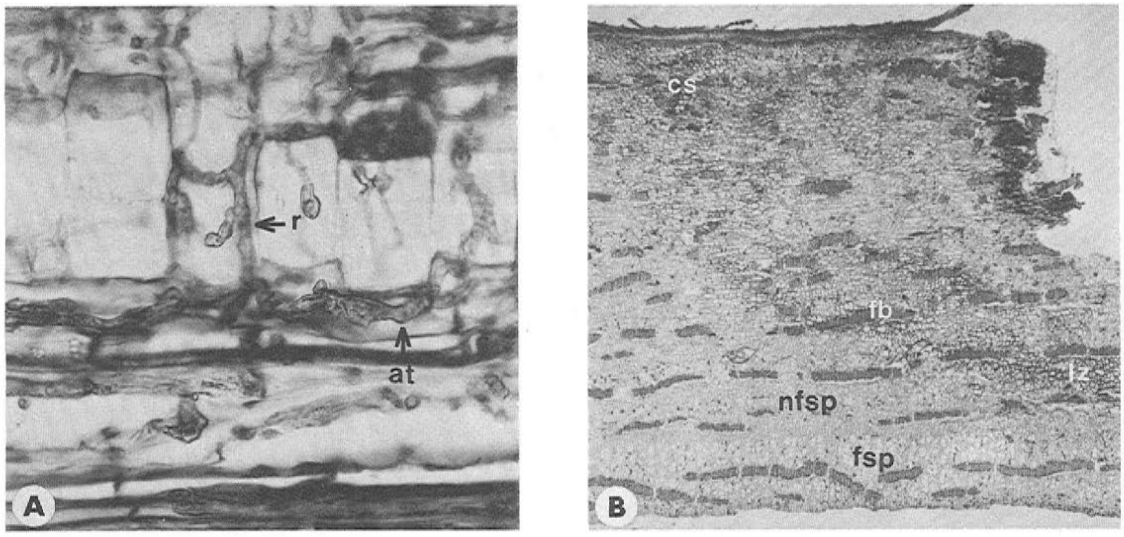


Figure 3. Micrographs of sections of chestnut blight cankers stained with safranin and fast green. Figure 3a. Radial section of an American chestnut stump sprout 12 days after inoculation with a virulent isolate of *Endothia parasitica*. Illustrates general appearance of hyphae in infected tissues. Note hyphae (arrows) in rays (r) and in axial phloem tissues (at) (x550). Figure 3b. Transverse section of a large, surviving American chestnut (the Fldg tree) 12 days after inoculation with a virulent isolate of *E. parasitica*. Inoculation wound is on the right top of section. Functioning (fsp) and nonfunctioning (nfsp) secondary phloem are evident. The infected tissues are surrounded by a dark-staining (safranin) zone of lignified cells (lz), which extends from the point marked by lz, through the noted fiber bundle (fb), and through the cortical sclerids (sc) to the outer periderm (x20).

The lignified zone was observed to form at the same time, about 8 to 10 days after inoculation, in all host-treatment combinations. The lignified zone appeared to be a wound periderm induction barrier; wound periderm formed next to it (Figure 4b). In the secondary phloem, wound periderm formation began about 2 to 4 days after lignification when the lignified zone was oriented periclinally. Wound periderm formation began in secondary phloem near the area of deepest canker penetration and progressed outward, taking about 20 days to reach the original periderm in the Fldg tree and the Chinese chestnut trees, and about 30 to 40 days in the American chestnut stump sprouts

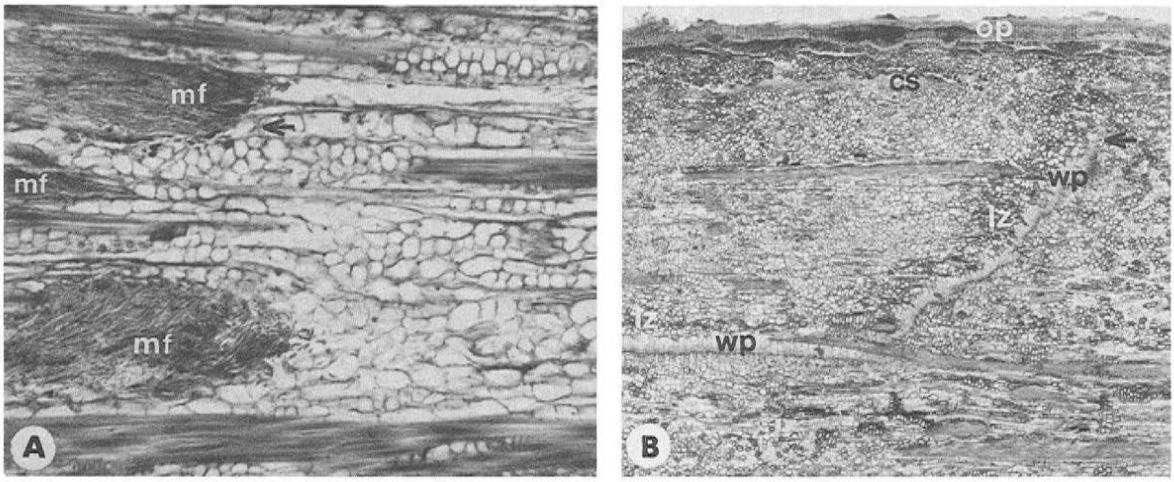


Figure 4. Micrographs of sections of chestnut blight cankers stained with safranin and fast green. Figure 4a. Mycelial fans (mf) of a virulent strain of *Endothia parasitica* in bark of an American chestnut stump sprout 23 days after inoculation. Note discoloration in front of bottommost fan, indicating cell death, and splitting of tissue in front of topmost fan. Arrow there points to a cell being physically crushed by the fan. Distortion of the bark tissues in this region is evident (x60). Figure 4b. Wound periderm (wp) formation next to a lignified zone (lz) in a large, surviving American chestnut (the Fldg tree), 53 days after inoculation with a virulent isolate of *E. parasitica*. Wound periderm does not extend (arrow) beyond cortical sclerids (cs) to the outer periderm (op) (x28).

(Figure 5). At any one place where wound periderm began to form, it took 4 to 8 days before phellem cells began to form and 8 more days before the number of phellem cells reached a maximum. The progress of phellem cell formation next to periclinally oriented lignified zones in secondary phloem is illustrated in Figure 6.

When the lignified zone was oriented anticlinally, the initiation of wound periderm formation was greatly delayed in any host-treatment combination. In secondary phloem, it began approximately 20 days after lignification instead of 4 days. Thus, anticlinally oriented lignified zones resulted in gaps in wound periderm. Anticlinally oriented portions of lignified zones were observed scattered among the samples in all bark tissues in all host-treatment combinations. They were especially prevalent where the lignified zone turned up to connect with the outer periderm (Figure 4b). The propensity of some branches (ca 10 years old) to initiate rhytidome formation appeared to augment the high frequency of gaps in wound periderm observed near the outer periderm.

Canker expansion always was accompanied by mycelial fan formation and expansion. Mycelial fans always penetrated through gaps in the wound periderm as far as could be determined. In the American chestnut stump sprouts, canker expansion beyond the lignified zone was first observed (at day 18 in 1978) with the V isolate and later (at day 28 in 1978) with the H isolate. On the stump sprouts, the cankers incited by the H isolate did not expand

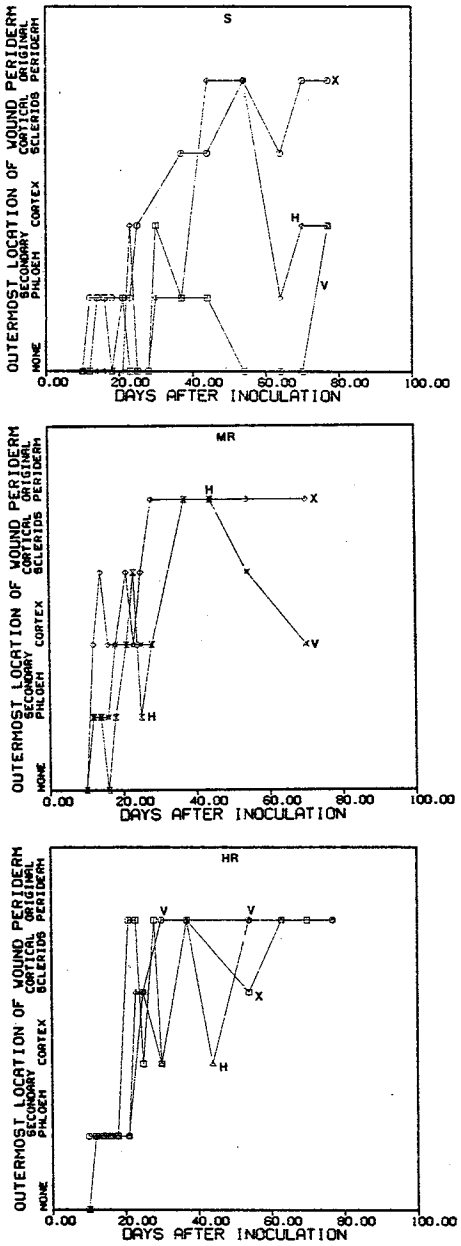


Figure 5. Time-course of region in bark tissue of outermost location of phellem or phelloderm of wound periderm in blight-susceptible (S) American chestnut stump sprouts, a moderately blight-resistant (MR), large, surviving American chestnut (the Fldg tree), and highly blight-resistant (HR) Chinese chestnut, cultivar Nanking, after inoculation on July 3, 1978 with virulent (V) and hypovirulent (H) strains of *Endothia parasitica*, or wounding with no inoculation (X). The scatter in the data occurred because each point represents a separate canker. When there were duplicate cankers on one day for the S trees, the canker with the outermost location is depicted.

as far as the cankers incited by the V isolate (Figure 2), and the H cankers eventually were surrounded by a wound periderm (Figure 5). There was no fan formation or canker expansion with the H isolate on the Fldg and Chinese chestnut trees. There was sporadic occurrence of canker expansion by the V

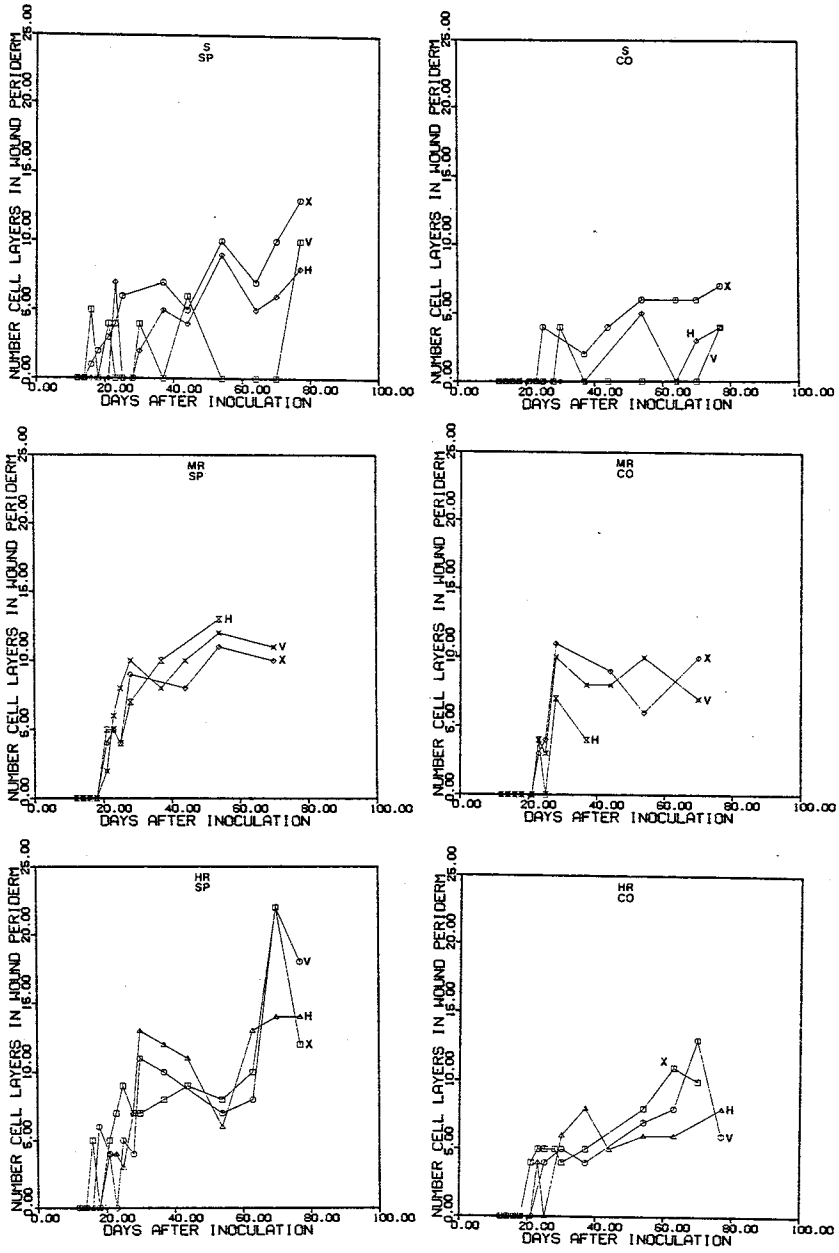


Figure 6. Time-course of change in the number of cell layers in phellem of wound periderm located in the secondary phloem (SP) and cortex (CO) of blight-susceptible (S) American chestnut stump sprouts, a moderately blight-resistant (MR), large, surviving American chestnut (the Fldg tree), and highly blight-resistant (HR) Chinese chestnut, cultivar Nanking, after inoculation on July 3, 1978, with virulent (V) and hypovirulent (H) strains of *Endothia parasitica*, or wounding with no inoculation (X). The scatter in the data occurred because each point represents a separate canker. When there were duplicate cankers on one day for the S trees, the canker with the greatest number of cell layers is depicted.

isolate on the Fldg and Chinese chestnut trees, with more instances of expansion in the Fldg tree. This difference in incidence of occurrence on canker expansion was the only difference we observed between the Fldg tree

and the Chinese chestnut trees. They did not appear to differ with respect to the rate or extent of wound periderm formation when fan expansion did not occur; the inoculated American chestnut stump sprouts, however, did show impairment in wound periderm formation. This is thought to have been due to formation and expansion of mycelial fans in the stump sprouts. The effects of resistance and hypovirulence appeared to be additive as far as rate and frequency of canker expansion were concerned.

The higher frequency of gaps in wound periderm near the outer periderm (Figure 4b), as compared to other bark regions, is part of the mechanism which gives rise to superficial cankers. When we observed canker expansion in the Fldg tree or in the Chinese chestnut trees, the mycelial fans commonly were underlain by a wound periderm. The fans in these two trees were short in comparison to those observed in the stump sprouts (less than 1 cm in length compared to lengths of up to 5 cm). This indicates that the fans expanded more slowly once expansion started, suggesting that the slow rate of fan expansion allowed wound periderm formation to keep pace. Bramble (1936) suggested that fan expansion in blight-susceptible American chestnut proceeds too rapidly for wound periderm formation to keep pace with it.

Biocontrol Tests

In tests using H strains to effect biocontrol of cankers incited by V strains (Hebard et al. 1981a), the rate of deceleration of growth in biocontrolled H+V cankers appeared to be inversely proportional (roughly) to the pathogenicity of the H strain (Hebard et al. 1979). The rate parameters in Hebard et al. (1979) should read cm/day instead of mm/day. Thus, H strains of slight or moderate pathogenicity gave rise to larger biocontrolled cankers than H strains with little pathogenicity. We observed, following dissection, that these larger biocontrolled cankers were superficial to a significant extent. Microscopical examination revealed that the superficial cankers were underlain by a wound periderm. The only instance of biocontrol (confirmed by isolation and pathogenicity tests of the isolates) of a V-alone canker (incited by the CR strain) which we observed occurred on a tree whose H+V canker had been biocontrolled by our most pathogenic H strain (EP-66). We suggest that, in Europe, superficial H cankers arise, in part, during the process of biocontrol of V cankers. We also suggest that the differences at vegetative compatibility loci between V and H strains govern the frequency with which V cankers are biocontrolled but not the rate of deceleration in canker expansion. The results of isolations and pathogenicity tests from biocontrolled cankers indicate that the spread of European hypovirulence factors can be monitored by determining the frequency of white isolates, as has been done in Europe (Grente and Berthelay-Sauret 1978; Mittempergher 1978).

Epidemics in Clearcuts

The main details of the epidemiological studies have been reported previously (Hebard et al. 1981b). Briefly, it takes 9 to 10 years after clearcutting for blight incidence on sprouts over 0.8 cm d.b.h. to increase from 20 percent, the level in forested areas, to 90 to 100 percent. This indicates that caution must be used in relying upon natural infection and mortality as a measure of blight resistance, especially when some blight-resistant trees are present; a blight-free tree at 10 years could well be an escape.

We also found that the increase in tree diameter after clearcutting probably is a major cause of the epidemics in clearcuts. The larger trees appear to allow a larger surface area of *E. parasitica* to develop than occurs in forested areas. This indicates that spread of V strains of *E. parasitica* is relatively inefficient at low population levels. It appears that a large host population surface is necessary for epidemics to occur. Arguing by analogy, we suggest that a large host (V strains of *E. parasitica*) population is necessary to obtain natural spread of hypovirulent factor(s). On the other hand, in a separate study at a 10-year-old clearcut site, chestnut sprouts treated with an H isolate of *E. parasitica* were overwhelmed by additional cankers before disease control could occur. Apparently, the epidemic at that site had progressed too far for biocontrol to be possible on these sprouts. Thus, a moderate population level for virulent *E. parasitica* (less than that at 10-year-old clearcuts but more than at forested areas) appears desirable. It may be possible to manipulate the size of *Endothia* population by regulating the mean diameter of American chestnut sprouts at regeneration sites. This appears to have occurred fortuitously in some European chestnut coppice where hypovirulence-associated blight remission occurred (Grente and Berthelay-Sauret 1978; Mittempergher 1978).

Schuepp (1961) measured canker growth and blight progress in Europe; both were slower than values reported in America (Hunt 1923; Gravatt and Gill 1930). We have developed a mathematical model (Hebard 1981) for assessing the effect of growth of individual cankers on disease progress. Using this model, we have found that the slower canker growth on European chestnut may be a principal cause of the slower rate of blight progress in Europe. It does not appear that hypovirulence was the cause of the slower disease progress and canker growth, since Schuepp's (1961) data were collected during the first 12 years after blight entered an area; Mittempergher (1978) reported that it takes about 15 years after blight enters an area before hypovirulence-associated disease remission begins. We suggest that the slower disease progress in Europe may have been a key factor in remission. Epidemics in clearcuts in the Appalachians probably could be prolonged by removing diseased sprouts and by removing (cutting) some large living trees so as to restrict inoculum production.

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SUMMARY OF HYPOVIRULENCE RESEARCH AT UTAH STATE UNIVERSITY

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ABSTRACT.--*The hypovirulent research program at Utah State University is directed toward understanding the biology of the hypovirulent factor and how this factor reduces the virulence of Endothia parasitica.*

Logan, Utah is a long way from the nearest American chestnut tree. Because of this distance, we in Utah will never directly enjoy the fruits of the successful control of chestnut blight. However, we feel that understanding the phenomenon of chestnut blight control by hypovirulence may lead to a wider exploitation of this phenomenon.

All of our research at Utah State University is directed toward understanding the biology of the hypovirulent factor. We are approaching this study from several different directions. Our two overall objectives are 1) to better understand the biological nature of the hypovirulent factor and 2) to understand how the hypovirulent factor reduces the virulence of *Endothia parasitica*.

Since other papers presented at this conference go into detail concerning our findings, I will only briefly mention the different approaches we are using to study these questions. One of our primary objectives is the cell-free transfer of the dsRNA associated with transmissible hypovirulence. This is a priority objective since direct proof of the role of this dsRNA in hypovirulence is needed. Our approach is to transfer the dsRNA encapsulated in liposomes into protoplasts of a virulent strain of the fungus.

In spite of the passage of many years since the demonstration that hypovirulence is cytoplasmically controlled, we are still uncertain about the biological nature of these cytoplasmic genes. As indicated above, dsRNA has been correlated with hypovirulence. Most researchers assume that the control of hypovirulence expression is determined by genes on the dsRNA. This is still speculation, however, since we have no direct evidence for this. Also we know little about how the dsRNA is packaged within the cell. On the basis of our work and that of Allan Dodds, we feel that the dsRNA is not packaged within a typical mycovirus. It appears that the dsRNA does not have a typical protein coat. The membrane-bound dsRNA containing particulate fraction that has been isolated from strain 113 is not a mycovirus, but is rather a membrane vesicle containing large amounts of the same carbohydrates that make up the fungal cell wall. This leads us to believe that the dsRNA

is packaged in cell-wall synthesizing vesicles. We do not know whether packaging of the dsRNA into these vesicles is a defense response of the fungus, or a dsRNA directed packaging phenomenon.

One way to determine the relationship of these vesicles to the dsRNA is to determine their role, if any, in dsRNA replication. A graduate student in our laboratory is doing his research on determining the site of dsRNA replication. He plans to use anti-body coupled with electron microscope techniques to determine where the replication occurs. He will particularly be seeking evidence of whether dsRNA is packaged in the vesicles at the same site that replication occurs.

The multi-segment nature of the dsRNA associated with hypovirulence is typical in that respect to mycoviruses. However, unlike mycoviruses, there is considerable strain-to-strain and intra-strain variability in the segmentation. This is unusual since differences in segmentation patterns are not consistently reflected by differences in hypovirulence phenotype. We are investigating the relationship between the dsRNA segments to determine whether they are redundant copies of each other, or whether they are each different from one another. We are using hybridization techniques for this study.

In addition to trying to better understand the nature of the hypovirulent factor, we are investigating how the presence of the dsRNA reduces the virulence of the fungus. We feel that the two most likely ways virulence is reduced are either by production of a gene product that interacts with the fungus, or by directly interacting with a virulence control site on the fungus genome. We are initiating research to test both of these hypotheses. One method we are using is to identify the translational products of the dsRNA. Another approach is to determine if there is a virulence control site on the fungus genome.

These various projects are all directed toward trying to answer the two questions raised above about hypovirulence: 1) What is its biological nature?, and 2) How does it affect the virulence of its fungal host? We currently have two people in addition to myself in our laboratory working on this project. Dr. Dane Hansen is studying the nature of the vesicles and how the dsRNA segments are related. Lee Barley, a graduate student, is studying replication of the dsRNA for his PhD research.

CHESTNUT BLIGHT: DEFENSE REACTIONS

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ABSTRACT.--*Anatomical studies showed that chestnut trees have the capacity to set boundaries to resist spread of infected bark and wood. Where portions of cambium remained alive after infection, xylem rays expanded into the bark and wood formed.*

Preliminary results of anatomical studies show that the pathogen in bark and wood is walled off, and wood is formed in bark by extensions of xylem rays.

Chestnut blight was viewed from CODIT, a model for compartmentalization of decay in trees. The CODIT perspective of stem and root diseases is that trees survive after injury and infection so long as they have the time, energy, and genetic capacity to recognize and compartmentalize injured and infected tissues rapidly and effectively and to generate enough new tissues to maintain the tree.

Materials and Methods

Stem sections--5 to 15 cm in diameter--with one or more cankers were received from Kentucky, 6; West Virginia, 60; North Carolina, 4; and Virginia, 12. Also, 10 sections of similar diameter, but without obvious cankers, were received from West Virginia. The stem sections ranged from 30 to 60 cm in length. The stems were first cut into disks, 1 to 3 cm in width. The disks were then sanded and studied under a dissecting microscope at 10 to 30 X. Selected samples, approximately 30, yielded one cm^3 subsamples of bark and wood for microtome sectioning 8 to 15 µm. The blocks were fixed in 5 percent formaldehyde. Sections were stained with 0.05 percent toluidine blue 0.

Results and Discussions

Where small portions of cambium remained alive after infection, xylem rays expanded outward into the bark. The bands of xylem seemed to start in wood that had the characteristics of a barrier zone. The xylem rays grew outward in the form of a pillar or cylinder. The cells formed a cambium that produced all wood components (Figures 1 and 2).

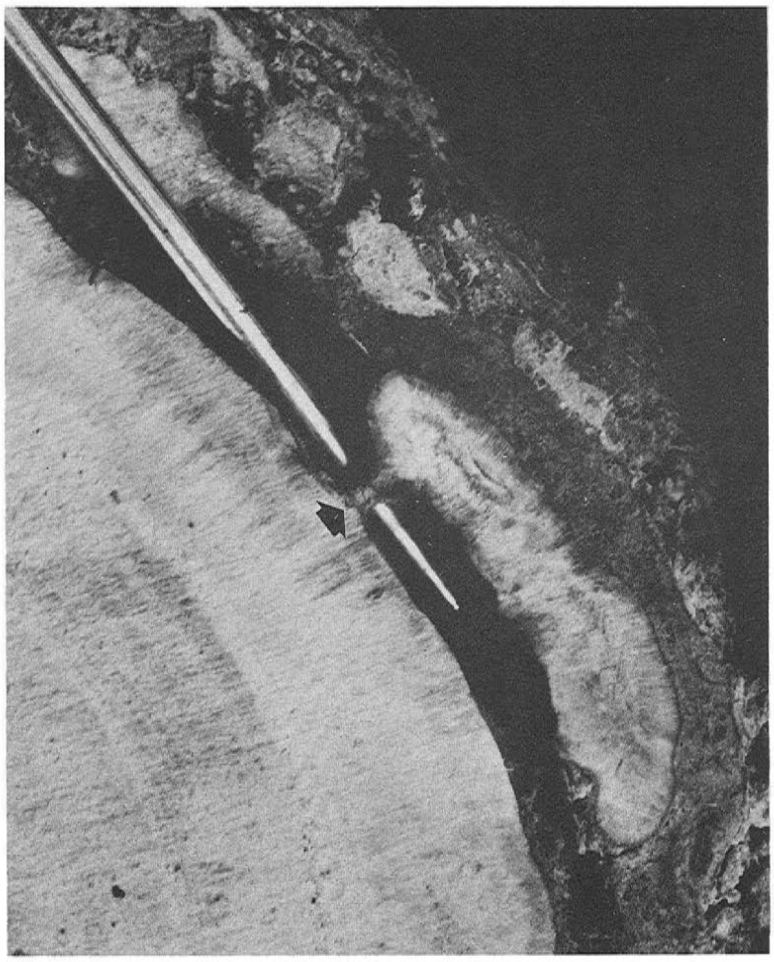


Figure 1. Extension of xylem ray forming wood in bark. The dead bark was removed to show the pillar or cylinder of xylem ray cells (arrow). A dissecting needle is beneath the pillar. The ray cells join to form a cambium that forms all wood components in the bark.

Wound periderms set boundaries for the infected bark. When the pathogen grew from the bark to the wood, the infected wood was walled off according to the CODIT model.

The results show that chestnut trees have the capacity to set boundaries to resist spread of infected tissues. The question is, why do most trees fail to effectively wall off infected tissues associated with wild strains of the pathogen, while many trees are fairly successful in effectively walling off infected tissues associated with the hypovirulent strain?

The key factors for survival are time, energy, and genetic capacity to recognize and compartmentalize infected tissues. Our results show that the trees have the genetic capacity to compartmentalize infected tissues. But compartmentalization does not start until the infected tissue is recognized.

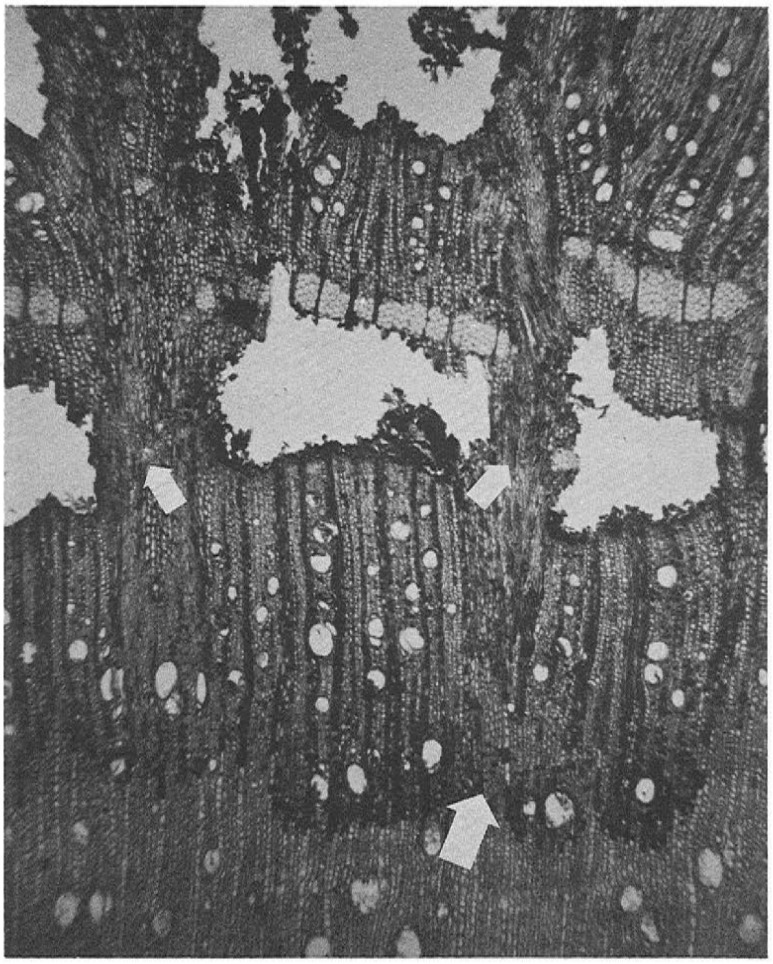


Figure 2. Portions of cambium that remain alive after infection form a barrier zone type tissue (large arrow). From this zone, ray cells divide rapidly to form pillars (small arrows) that extend into the infected bark. The xylem ray cells then form a cambium that produces all components of wood.

Time may be the key factor. One possible explanation is that the wild strains spread rapidly in outer bark first before they spread inward and are recognized by the cambium. The hypovirulent strains may spread slowly in outer bark, but rapidly inward to the cambium. If this is so, it would explain the usual ellipsoid shape of the hypovirulent-incited cankers. Such shapes of cankers or dead areas are typical of drill wounds where a portion of cambium is killed quickly.

HYBRID CHESTNUTS AT THE LESESNE FOREST, VIRGINIA

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ABSTRACT.--*Approximately 12,000 hybrid chestnut seedlings were planted at the Lesesne State Forest, Nelson County, Virginia, between 1969 and 1975. Most of the trees were grown from open-pollinated seed of the best hybrid chestnut trees available based on tree form and apparent blight resistance. Seed was collected from 46 different trees or tree collections at several locations, with much of it derived from selections of breeding efforts at the Connecticut Agricultural Experiment Station. Outstanding individual trees in the Lesesne planting were selected in the spring of 1972, 1975, and 1980. Form, vigor, and blight resistance of several selections have been impressive to date. Efforts to propagate selections and future plans for seed orchards and controlled pollinations were discussed.*

A classical approach to counteracting a serious plant pathogen of crops is to breed a resistant variety. Resistant species of chestnut exist and breeding for a blight-resistant, American-like chestnut has proceeded for some 60 years with varying degrees of intensity (Jaynes 1972; 1978). However, it has not been a massive effort, at least from the perspective of growing large segregating populations of several thousand individuals and carrying the process through several generations of trees. Chestnut breeders had assumed their goals could be reached with relatively small populations, something which in hindsight we can now question. To date, no single hybrid has been selected which appears to be fully comparable in growth characteristics to the American chestnut and is also highly resistant to the blight. Progress has been made, but the apparent combination of multi-genic control and/or linkage of good form and susceptibility, versus poor form and resistance, indicate that larger populations and more generations of selection are needed to attain the goal.

The Lesesne Planting

The encouragement and financial support of Arthur and Anne Valk has resulted in a substantial planting of hybrid chestnut on land donated to the State of Virginia and now called the Lesesne State Forest in Nelson County. R. A. Jaynes and associates at the Connecticut Agricultural Experiment Station

supplied the seed or seedlings and T. A. Dierauf and associates with the Virginia Division of Forestry planted and cared for the trees (Dierauf 1977; Jaynes 1971).

During 1969 to 1976, approximately 11,500 chestnut seedlings from 46 different seed sources were planted at the Lesesne Forest (Table 1). Most were field planted as 1-year-old seedlings, although some just-germinated seed in tubes and 2-year-old trees were also planted. Open pollinated seed was the rule, but a few controlled crosses were included. Most of the seed parents were hybrids selected for good form, vigor, and blight resistance. Some American and Chinese sources, as well as hybrid nut-tree selections, were also included for comparative purposes.

Over half of the seed parent trees were located in six different Connecticut Agricultural Experiment Station plantings. Many of these trees, such as those labeled WdsL (Table 1), were in situations where the pollen parent would also have been a tree selected for improved form, vigor, and blight resistance, the poorer trees having been culled. Five of the seed sources were trees in cooperative test plots established by J. D. Diller between 1947 and 1955 (Berry 1980). The 'Clapper' chestnut, a hybrid by R. B. Clapper grown in the Carbondale, Illinois plot was the best known of these trees.

Cultural Notes

The Lesesne planting site was an abandoned farm that, prior to planting, was cleared of brush and boulders and then fenced to keep cattle out. In 1969, the first 1,100 trees were planted at 1.2 x 4.9 m; subsequent spacing of all trees was 1.2 x 2.4 m. There has been no thinning. The weaker and blight susceptible trees will be suppressed by the more vigorous hybrids.

Combinations of contact and residual herbicides were spot sprayed around newly planted trees and annually reapplied for 2 to 3 years until the trees were well established. Annual mowing was also done the first few years after planting to control competing vegetation. Woody invaders such as *Robinia*, *Ailanthus*, and *Vitis* were periodically killed with treatments of a phenoxy herbicide. Fertilizer (0.11 to 0.23 kg 10-10-10) was applied to all newly planted trees and reapplied annually for up to 3 years.

Survival of trees has been good. However, on at least three occasions, 1971, 1972, 1978, and to a lesser degree 1974, trees were injured by what appeared to have been an early hard freeze in the fall. Although the planting site is high with good air drainage, there is a mountain to the north that rises another 305 m. Cold air on clear, still nights drains down the slope. Bark on the lower portion of the main stem of young trees was killed. Affected trees occur in irregular patches with the pattern more related to topography than to tree genotype.

Seasonal fluctuations in rainfall have affected growth. For instance, in 1980 and 1981 the growing seasons were very dry and growth was less. A notable event, but one that had little effect on the trees, was 51 to 69 cm rainfall that occurred August 19 to 20, 1969, when Hurricane Camille went through.

Table 1. Pedigree, number, and characteristics of seed parents for chestnut seedlings planted at the Lesesne State Forest between 1969 and 1976

Seed source		Seed parent characteristics		Number
Identification	Pedigree[1]	Blight resistance	Tree form	planted trees
'Sleeping Giant'	C•JA	Good	Good	1029
C3	(JP•J)C	Good	Good	126
C4	C•JA	Good	Good	54
C9	C•JA	Good	Good	1188
C13	C•hybrid	Good	Excellent	959
C14	C•JA	Good	Good	1101
'Clapper'	CA•A	Fair	Excellent	129
WdsL R11T7	C•JAxA	Fair	Excellent	550
WdsL R11T8	C•JAxA	Fair	Excellent	302
WdsL R12T10	C•JAxA	Fair	Excellent	931
WdsL Misc.	C•JA's	Variable	Excellent	320
1-71 Controlled Cross	C3•Clapper	Good	Excellent	51
3-70 Controlled Cross	Sleeping GiantxClapper			62
OTR7T8	J•AC	Good	Good	259
Broker, Cheshire	C•JAxC•JA	Good	Good	179
Easton, Bridgeport	C•JAxC•JA	Fair-good	Good	309
#40 Norfolk	JA•hybrid	Fair	Excellent	131
NH R9T6	JA•C	Good	Good	27
NHB (4 trees)	C•JA's	Good	Good	1245
NHB	(J•JA)C	Good	Good	180
R9T12	AC•C	Good	Excellent	126
R10T10	CA	Poor	Excellent	
R4T10	JA	Poor	Excellent	23
R10T12	CA	Poor	Excellent	563
IL-Mix	CA's	Variable	Good	28
B-71	C	Good	Good	7
B-70	C	Good	Good	14
B-1	C	Good	Good	5
IL-Chin	C	Good	Good	15
'Eaton'	C-hybrid	Good	Orchard	267
RR R8T5	CJ	Good	Good	72
Grassman	AC	Poor	Excellent	32
Grassman	JA	Fair	Good	79
Grassman	A	Poor	Excellent	32
Siebentritt	A	Poor	Excellent	17
Sauber	A	Poor	Excellent	62
Inshi	C	Good	Orchard	25
Red Win	C	Good	Orchard	25
Hemming C	C	Good	Orchard	40
Quin. C orig.	C	Good	Orchard	41
Quin. C reg.	C	Good	Orchard	25
V188 Baldwin NH	C	Good	Good	405
NHB 58602	C	Good	Good	379
OxC	OC	Fair	Orchard	60
R23T12	CS•C	Good	Orchard	44
1-70 Controlled Cross	CS•CSxC	Good	Orchard	24
			Total	11,542

[1] C=*Castanea mollisima;* J=*C. crenata;* A=*C. dentata;* P= *C. pumila;* S=*C. seguinii*

Selection

Our first attempt to select trees was in the spring of 1972. Approximately 1,000 3-year-old trees were evaluated and 15 selected as superior for form and vigor. Three years later five of the selected trees had been killed back by cold, four had lost vigor and form, and six maintained desirable form and vigor. We knew it was premature to select for blight resistance, but we also learned that 3-year-old chestnut trees are too young to select for form and vigor.

In March 1975, we examined the 1969 to 1971 hybrid plantings of about 4,700 trees and selected 106 trees for blight resistance with desirable growth and form. These trees were reevaluated in March 1976. Only 43 of the original 106 trees met the standard of the year before. Loss of apical dominance was the biggest problem. In March 1980, only 18 of the original 106 trees were still as promising as they had been in 1975. We then selected 18 additional trees of merit. When the same planting was evaluated again, two growing seasons later in the fall of 1981, only three of the original trees selected in 1975 and five of the 18 trees selected in 1980 were still rated as good when first selected.

Loss of apical dominance (narrow forks and multiple leaders) continued to be a big problem. Selected trees have been eliminated because their terminal growth has not been sufficient to keep them in the dominant crown canopy. The major cause, however, has been chestnut blight. Many of the original selections were girdled and many more have severe cankers which eliminate them from consideration for future breeding. Practically all of the remaining selected trees have been challenged by chestnut blight; that is, they have at least superficial cankers.

Although all the progeny at Lesesne have not been evaluated, it is clear that offspring of blight susceptible trees such as 'Clapper' and WdsL selections (Table 1) are, in general, highly susceptible to the blight even when the seed parent was crossed by neighboring trees that were blight resistant. The original Clapper tree was girdled and killed by the blight in 1977. It had obtained a height of 21 m and d.b.h. of 36 cm in 25 years. One of its offspring was the most impressive tree in the Lesesne planting in 1980. This seedling had a straight central leader and was 11 m tall at 10 years, but now in its 12th year is severely blighted.

One of the most promising seed parents for production of seedlings with good form, vigor, and blight resistance is C13. It is a Chinese chestnut cross of unknown parentage growing in a small mixed hybrid planting at Redding Ridge, Connecticut. Of 16 new selections made in the fall of 1981, one-half were C13 seedlings whereas only 20 percent of the population examined were of the C13 source.

We are encouraged by the possibilities of selecting blight resistant hybrid chestnuts that would compete satisfactorily in forest tree plantings. However, as previously stated, no selection appears fully comparable in growth characteristics to the American chestnut and is also highly resistant to the blight. Burham (1981) has recently proposed that there is still hope for breeding a blight resistant American chestnut by recurrent backcrossing from the resistant species to the American chestnut.

However, our experience at Lesesne and some 25 years experience in breeding and growing chestnut hybrids suggests that adequate field resistance may never be recovered by relying on crosses with pedigrees that are predominantly *Castanea dentata*. Finite inheritance data are not available so the point is not conclusive. Because so few resistant progeny results from a cross of one susceptible and one resistant parent (e.g. Clapper x Sleeping Giant), we prefer to work with the best of the blight resistant trees.

Future Plans--Seed Orchards

In March of 1980, scions from eleven of the most promising selections at Lesesne were propagated by grafting dormant buds on germinating nuts (Jaynes 1980). Our intentions are to vegetatively propagate the best hybrids and establish two seed orchards, one in Virginia and one in Connecticut. Two kinds of nuts will be obtained from these seed orchards, one will be from controlled crosses and the other from open-pollinated nuts. The orchards will be isolated so open-pollinated seed would result from natural crossing among the grafted selections. To the extent that labor and resources allow, controlled crosses will be made, but even without controlled crosses the open-pollinated seed should be genetically better than what we have available now.

Obviously, if adequate field control of chestnut blight on American chestnut is obtained in the United States, then the effort to breed a blight resistant hybrid will have been redundant. However, the lack of demonstrated natural spread of hypovirulent strains in the field to date suggests that hybrids may yet play a role if a higher level of host resistance is required than that in pure *C. dentata*.

The long-term goal is to develop a true-breeding strain of chestnut with favorable growth characteristics and resistance to the chestnut blight fungus. It was not anticipated that hybrid trees now growing at Lesesne would meet the final goal, but these trees give us an opportunity to select improved individuals of a family and thus move closer to the ultimate goal.

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DISEASE INCIDENCE, SYMPTOMATOLOGY, AND VEGETATIVE COMPATIBILITY TYPE DISTRIBUTION OF *ENDOTHIA PARASITICA* ON OAK AND CHESTNUT HOSTS IN NORTH CAROLINA

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ABSTRACT.--*American chestnut, scarlet oak, white oak, and post oak were identified as hosts of Endothia parasitica in the North Carolina mountains and Piedmont. Symptomatology of the oak hosts, and disease incidence by region are given. A diversity of vegetative compatibility (v-c) types were collected from each host. Similar specific v-c types were found on the different hosts, however the relative frequencies varied between hosts. Scarlet oak, because of its range, has the greatest potential for use in hypovirulence research.*

In the United States, *Endothia parasitica* has been reported on several species of oaks including: scarlet *Quercus coccinea*, post *Q. stellata*, and white *Q. alba* oaks. Unfortunately, little has been published concerning *E. parasitica* on these hosts other than confirmation of etiology and descriptions of symptom expression (Anderson and Babcock 1913; Rankin 1914; Clapper et al. 1946; Ham 1967). A more complete knowledge of these oak hosts is needed to determine their importance as virulent inoculum sources and as potential reservoirs of hypovirulent (H) strains of *E. parasitica*. This study was designed to determine the symptomatology, regional disease incidence, and patterns of vegetative compatibility (v-c) types on the hosts of *E. parasitica* in the North Carolina Piedmont and mountains.

Materials and Methods

Mountain and Piedmont Surveys. These two surveys included all 21 mountain counties of North Carolina, and 12 Piedmont counties selected for their large oak populations. Gridded county highway maps were used to locate 6 randomly chosen plots in each county. In the field, each plot was described and systematically subdivided into 5 subplots, employing the methodology of the U. S. Forest Service Renewable Resources Evaluation Project (1977). Subplot boundaries were established with a 10-factor basal area panama tube. Within each subplot, all American chestnuts, *Castanea dentata*, and all oaks with a minimum 2.5 cm d.b.h. were identified and examined for the presence of *E. parasitica*. Each infection was characterized and appropriate symptom or sign material collected for laboratory isolation. Suscepts between subplots and additional stands of suscepts identified by local U. S. Forest

Service personnel were also investigated, as time permitted. Several additional collections of *E. parasitica* were made to supplement the isolate base for v-c testing. The first of these involved periodic sampling of American chestnuts along the entire length of the Blue Ridge Parkway (BRP) in North Carolina. A second set of isolates, obtained from scarlet, post, and white oaks, was amassed in a limited survey at Stone Mountain State Park, North Carolina. The last group of isolates (to be called Mixed survey) represents cultures obtained on various trips throughout the state during 1979 to 1980.

Vegetative compatibility testing. *Endothia parasitica* was best isolated from infected host tissue collected from either the canker margin (American chestnut), loosened bark (post oak), or involuted inner bark tissues (scarlet and white oaks). Samples were flame sterilized, plated onto potato dextrose agar (PDA) and incubated on a laboratory bench at room temperature. *Endothia parasitica* usually appeared 2 to 3 days later. Isolates were then transferred to PDA slant culture for long-term refrigerated storage.

Vegetative compatibility types were determined using the method of Anagnostakis (1978). Survey isolates were first grown on PDA amended with 100 mg/l methionine and 1 mg/l biotin. Mycelial plugs were removed from culture margins and paired in new plates of the same media with known v-c testers supplied by the Connecticut Agricultural Experiment Station (v-c types 1 to 63). These plates were incubated at 25 C in the dark and later examined for vegetatively compatible (merge) or incompatible (barrage formation) reactions. Each survey isolate was initially paired with ten v-c testers. Isolates that were not compatible with any of these testers were then paired with an additional 10 v-c testers. After the third series in this manner, all remaining survey isolates were tested against all remaining v-c testers. At least three pairings were made between each unknown and v-c tester.

Results

Mountain and Piedmont surveys. Eleven species of oak were examined during the course of this study, eight of which were not infected by *E. parasitica*. These species, and the total number of stems examined were: northern red oak *Q. rubra*, 424; chestnut oak *Q. prinus*, 353; southern red oak *Q. falcata*, 162; black oak *Q. velutina*, 117; water oak *Q. nigra*, 22; willow oak *Q. phellos*, 13; blackjack oak *Q. marilandica*, 12; and chinkapin oak *Q. muehlenbergii*, 1. American chestnut, scarlet oak, post oak, and white oak were identified as hosts of *E. parasitica* in this survey (Figures 1, 2 and 3). Incidence of infection varied by species and region (Table 1). Disease incidence for all hosts was slightly higher in the mountains (11.4 percent) than in the Piedmont (10.5 percent) or in both regions combined (11.1 percent).

American chestnut was found primarily as small stump sprouts in the mountain counties. The low incidence of infection (17.0 percent) probably reflects the large number of these sprouts and their associated juvenile resistance. There was no evidence, based on visible symptomatology or subsequent cultural characteristics, that any cankers on American chestnut contained H strains of *E. parasitica*.



Figure 1. Symptomatology of *Endothia parasitica* on oak. Swollen butt of scarlet oak showing multiple cankers and rough involuted bark.

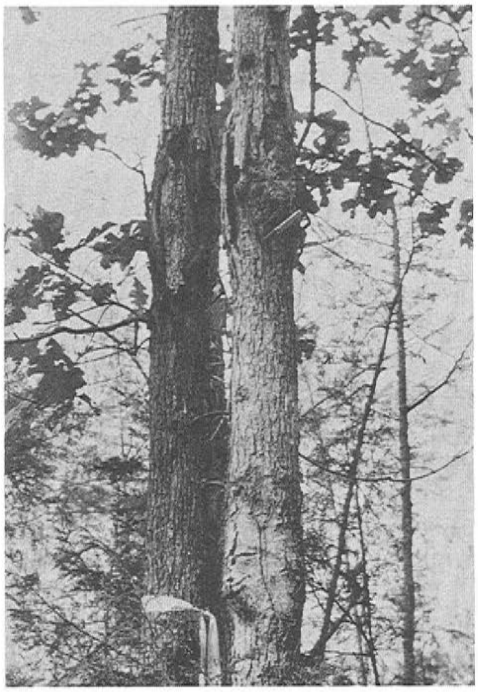


Figure 2. *Endothia* canker of post oak. Note cracked bark and various degrees of callus formation.



Figure 3. *Endothia parasitica* on white oak.

Table 1. Percent incidence of *Endothia parasitica* on various hosts in a 33-county survey of North Carolina

Host	Piedmont[a/]	Mountains	Regions combined
American chestnut	0.0 (1)	17.0 (866)	17.0 (867)
Scarlet oak	25.9 (174)	8.6 (406)	13.8 (580)
Post oak	14.5 (69)	50.0 (2)	15.5 (71)
White oak	1.0 (310)	1.0 (364)	1.0 (674)
Total	10.5 (554)	11.4 (1638)	11.1 (2192)

[a/] Number in parenthesis is total trees.

Infected scarlet oaks were most often characterized by the swollen butt condition, as first described by Ham (1967). Basal cankering by *E. parasitica* resulted in various degrees of basal swelling, callus formation, involution of bark, and distortion of wood tissues beneath cankered areas. Infected tissues were sometimes localized, but more typically the entire basal circumference was involved. Pycnidia, (and rarely perithecial stromata), were usually present in bark crevices. Orange mycelial fans were always present. *Endothia parasitica* was also isolated from small bole cankers. These cankers, which never exceeded a few centimeters in diameter, were sometimes found on trees that also exhibited swollen butt (Table 2). Infected scarlet oaks were found in the Piedmont and mountains, although infected trees were almost three times more prevalent in the Piedmont. In both regions, diseased trees were usually found in groups, rather than as randomly scattered trees. The d.b.h. of infected trees ranged from 11.9 to 78.7 cm inches with a mean of 31.2 cm.

Table 2. Symptomatology of *Endothia* canker on Scarlet oak in North Carolina from the 1979-80 33-county survey

	Number of Trees		
Symptomatology	Piedmont	Mountains	Regions combined
Swollen Butt	31	31	62
Bole cankers	5	3	8
Both symptoms	9	1	10
Total	45	35	80

Post oak was found primarily in the Piedmont, where 14.5 percent of the total 69 trees exhibited one to numerous irregular bole cankers. These cankers were typified by cracked or missing bark, and an abundance of fungal tissue. This tissue was sometimes so extensive that the limits of individual cankers could not be delineated. Examination of the canker face, which was often surrounded by callus, and the interior portions of loosened bark revealed numerous pycnidia and rarely perithecial stromata. Completely callused cankers were sometimes observed. Infected post oaks, like scarlet oaks, were usually found in groups. The d.b.h. of the infected trees ranged from 16 to 53.1 cm with a mean of 30 cm.

The disease incidence of white oak was 1 percent in both the mountains and the Piedmont. *Endothia parasitica* on this host species was associated with a range of symptomatology including: basal swelling and cracking, gall formation, and wounds within stem crotches. Orange fan material was always present.

Vegetative compatibility testing. In this study, 278 isolates of *E. parasitica* were obtained from 247 trees. Some of these isolates, even upon repeated testing, showed compatible reactions with more than one of the v-c testers. It was decided for the purposes of this study, to list each compatible reaction as a separate v-c type. Thus, 1 survey isolate could be placed with 2 or more different v-c types. Following this procedure, our 278 isolates yielded 332 compatible reactions with 48 different v-c testers. This data, summarized by host species in Table 3, shows that a diversity of v-c types were collected from each host species. An additional 55 isolates did not show compatible reactions with any of the v-c tests.

Table 3. Distribution of vegetative compatibility (v-c) types among hosts of *Endothia parasitica* in North Carolina, 1979 to 1981

Hosts	Trees	v-c typed isolates	Number of different v-c groups[a/]
American chestnut	117	163	34
Scarlet oak	103	134	36
Post oak	12	16	11
White oak	10	11	8
Chestnut species	3	6	6
Oak species	2	2	2
Total	247	332	

a/ v-c groups as delineated by the Connecticut Agricultural Experiment Station.

The v-c types found most often on American chestnut were similar to those found most often on the other hosts of *E. parasitica* (Table 4). However, the relative frequency among these v-c types did vary between hosts. The number

Table 4. Frequency of the most common vegetative compatibility (v-c) types on hosts of *Endothia parasitica* in North Carolina, 1979 to 1981

Host				
American chestnut (163 isolates)[a/]	Scarlet oak (134 isolates)	Post oak (16 isolates)	White oak (11 isolates)	All hosts (332 isolates)
43 (28)[b/]	9 (33)	9 (4)	9 (4)	9 (54)
24 (18)	19 (11)	18 (2)		43 (37)
9 (13)	24 (7)	37 (2)		24 (25)
15 (10)	43 (7)			19 (24)
19 (10)	42 (6)			15 (17)
44 (10)	44 (5)			44 (16)
				8 (13)

[a/] Listed by vegetative compatibility (v-c) type and number of trees yielding that type.

[b/] This v-c number corresponds to those assigned by the Connecticut Agricultural Experiment Station.

of different v-c types by survey was: Mountain (26 v-c types), Piedmont (26), Blue Ridge Parkway (32), Stone Mountain State Park (16), and Mixed collection (22). If all of the collections made in the mountain region are considered as a unit (Mountain, Blue Ridge Parkway, and Stone Mountain surveys), then 42 different v-c types are represented. The most common v-c types found in these collections are listed in Table 5.

Scarlet oak was the only host species with large numbers of infected individuals in both the mountains and Piedmont. The v-c data for this species were examined for regional differences in v-c type distribution. In the Mountain survey, 53 isolates were obtained from 40 trees. Vegetative compatibility testing identified 21 different v-c types. These data were similar to that obtained from the Piedmont, which yielded 55 isolates from 46 trees representing 25 v-c types. The ratio of number of trees to v-c types was 1.0:1 and 1.84:1 for the mountains and Piedmont, respectively. In both regions, the two most common v-c types were v-c 9 (mountains: 9 isolates, Piedmont: 5 isolates) and v-c 19 (15,5). Thus, there did not appear to be large regional differences in v-c type distribution. Multiple isolations made on 15 scarlet oaks showed that 9 of these trees contained more than 1 v-c type per tree. Vegetative compatibility types differed between bole cankers and swollen butt on the same tree and within a single swollen butt. Examination of v-c type distribution within a small geographic area (Stone Mountain State Park survey) showed that many v-c types were present (12 v-c types on 10 trees). This trend was also noticed with American chestnut on the BRP survey.

Table 5. Frequency of the most common vegetative compatibility (v-c) types of *Endothia parasitica* by survey in North Carolina, 1979 to 1981

Mountains[a] 97 isolates	Piedmont 66 isolates	Blue Ridge 100 isolates	Stone Mountain 23 isolates	Mixed[b] 46 isolates	Mountain Region[c] 220 isolates
9 (17)[d]	9 (20)	43 (15)	9 (6)	43 (7)	43 (29)
43 (12)	19 (6)	24 (10)	37 (2)	9 (6)	9 (28)
4 (9)	42 (5)	15 (7)	43 (2)	24 (5)	24 (18)
15 (7)	56 (5)	44 (7)		19 (4)	15 (15)
19 (7)	41 (3)	19 (6)		34 (3)	19 (14)
24 (7)		8 (5)		44 (3)	44 (12)
8 (5)		9 (5)			8 (10)
					4 (10)

[a] Listed by vegetative compatibility (v-c) type and number of trees yielding that type.

[b] Isolates obtained on several trips to different sections of North Carolina.

[c] Total of Mountain, Blue Ridge Parkway, and Stone Mountain State Park surveys.

[d] The v-c numbers correspond to those assigned by the Connecticut Agricultural Experiment Station.

Discussion

While white oak was only rarely (1 percent) infected by *E. parasitica*, diseased scarlet and post oaks were found in significant numbers in North Carolina. The disease incidence on post oak, found only in the Piedmont, was 14.5 percent. This figure compares very closely with the results of Bryan (1960) who, in a survey of the North Carolina, South Carolina, and Georgia Piedmont, found 14.3 percent of the post oaks infected. The observation of pycnidia and the perithecial stromata on infected trees, and the collection of viable spores of *E. parasitica* in preliminary stem flow experiments, indicates that post oak should be considered as a source of virulent inoculum of *E. parasitica*. Post oak appeared to be the most susceptible of the oaks in this survey, because cankers can coalesce and cause mortality. The ability of H strains of *E. parasitica* to heal virulent cankers on this host needs to be determined. However, as post oak is not often found intermixed with American chestnut, its role in the restoration of the chestnut may be limited.

While *E. parasitica* did not appear to be an important mortality factor on scarlet oak in this study, it must be considered as an important source of

virulent inoculum. Infected trees, usually with asexual or sexual fruiting structures, were found in significant numbers in both the Piedmont (25.9 percent) and the mountains (8.6 percent). Cross inoculation studies designed to test the relative pathogenicity of *E. parasitica* obtained from scarlet oak and the other hosts of the pathogen, are in progress at Duke University. The widespread distribution of scarlet oak may offer opportunities for disease control using hypovirulent strains of *E. parasitica*. If hypovirulent strains of the pathogen could be established on scarlet oak, they would be well situated to serve as hypovirulent inoculum reservoirs. Whether these inoculum sources can be established or maintained on scarlet oak is unknown at present. The rough bark that typifies swollen butt, and the involutions and irregular nature of the infection will make establishment and evaluation of H strains difficult.

A diversity of v-c types were collected from each host of *E. parasitica*. Similar results have been reported by other researchers working with American chestnut in the United States (Anagnostakis and Waggoner 1981; MacDonald and Double 1978). The v-c types isolated most frequently from American chestnut in this study were similar to those isolated most frequently from other hosts of *E. parasitica*. The relative frequencies of these v-c types however, did vary between hosts. Studies with scarlet oak, found in both the mountains and Piedmont, showed similar degrees of v-c type diversity and similar specific v-c types in these two regions. More than one v-c type was sometimes present within a single scarlet oak with multiple infections or from several trees within a limited geographical area. These results suggest that any treatment with H strains of *E. parasitica* on infected oaks should utilize batch mixtures of several v-c types.

It is not known why some of our isolates gave compatible reactions with more than one v-c tester. Presumably, these results identify v-c types that are genetically related. This theory is supported by the fact that certain patterns of v-c types were repeatedly involved in these multiple reactions.

We feel that research on the oak hosts of *E. parasitica* should continue. While it is true that scarlet and post oaks are not commercially important species, they do play an important role in the maintenance of inoculum levels of *E. parasitica* in North Carolina. Furthermore, the potential for conversion of their cankers to hypovirulent forms of the pathogen needs to be evaluated. Scarlet and post oak may be important in the control of *E. parasitica* in North Carolina.

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ENDOTHIA PARASITICA ON NUTS OF CASTANEA DENTATA

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ABSTRACT.--*The chestnut blight fungus was reported on nuts of Castanea sativa in Pennsylvania in 1915. Visual examination of germinating American chestnuts in the spring of 1981 indicated that 38 percent, 88 of 234, were infected with Endothia parasitica. These were collected from trees at Lockwood Experimental Farm, Hamden, in an area where cytoplasmic hypovirulent strains have been introduced. Our initial identification was based on the presence of orange mycelial fans just below the epidermis of the nut shell and on fruiting pustules that erupted through the shell. Apparent E. parasitica was isolated free of contaminants from 26 of 41 infected nuts tested. Fourteen of these isolates displayed varying degrees of abnormal morphology in culture. Two of the isolates are hypovirulent based on a pathogenicity test on American chestnut. Several isolates are being tested for double-stranded RNA. Infection of the nut shell does not appear to affect germination or health and vigor of seedlings through the first growing season. The means by which these nuts become infected and possible significance for spread of hypovirulent strains is being examined.*

The chestnut blight fungus, *Endothia parasitica*, is normally spread by the transport of ascospores and conidia and perhaps by mycelial fragments. Long distance spread may result from the physical movement of infected stems and subsequent dispersal of spores and mycelium. Infection arising from nuts has generally been assumed to be by surface contamination which could be eliminated by surface disinfection (Lanza 1950). However, more than a half century ago, one investigator (Collins 1913; 1915) reported finding infected chestnuts lying on the ground in Pennsylvania and Delaware. The tree species, identified only in the second paper, was *Castanea sativa*, the European chestnut. Infections of *E. parasitica* on American chestnuts, *C. dentata*, have not been reported.

Methods and Results

1980 Harvest

In the fall of 1980, we collected American chestnuts from field-planted 14-year-old trees at the Lockwood Farm, Hamden, Connecticut, in an area where cytoplasmic hypovirulent strains have been introduced. The nuts were stratified in peat moss and kept at 4 C during the winter, and in February were sown in flats of moist peat moss in the greenhouse.

Visual examination of the germinating nuts five and nine weeks after sowing indicated that a total of 38 percent (88 of 234) were infected with *E. parasitica*. This determination was based on the presence of orange mycelial fans just below the epidermis of the nuts and on fruiting pustules that had erupted through the shell (Figure 1).

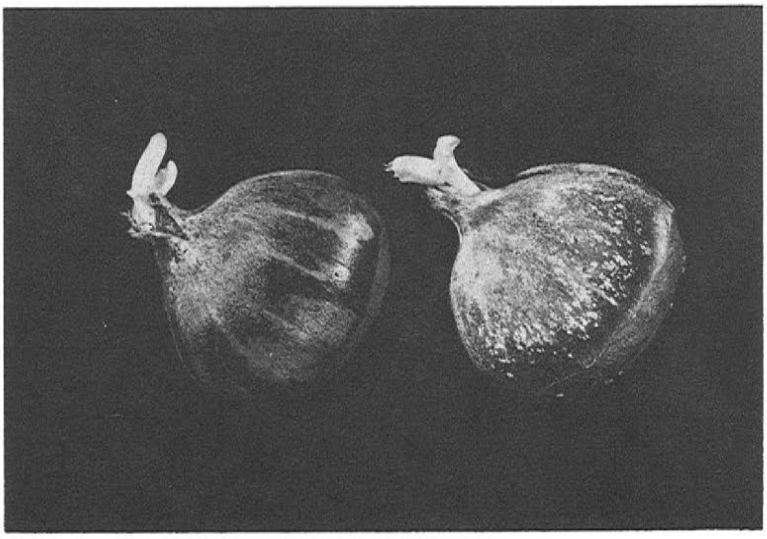


Figure 1. Nuts of *C. dentata* infected with *E. parasitica*: left mycelial fan, right pycnidia erupted through shell.

Twenty-six nuts with fruiting bodies were sampled and *E. parasitica*-like isolates were obtained from all of them. No *E. parasitica* was recovered from the 15 nuts with only mycelial fans, but this may have been due to contamination. Two of the nuts with mycelial fans later produced fruiting bodies and *E. parasitica*-like isolates were recovered from these also.

Twenty-six isolates were examined for morphology and pathogenicity. Twelve were classed as morphologically normal while the other 14 ranged from almost normal to highly abnormal in appearance. Two dsRNA extractions were run on 7 of the most abnormal isolates; no dsRNA was detected.

The 26 isolates were inoculated into American chestnut trees in May 1981. Measurements taken in September indicated that three of the isolates were much less pathogenic than normal and two other isolates had intermediate pathogenicity.

Seedlings which were produced from the infected nuts were field planted during summer 1981. Observations in late fall indicated that tree vigor and survival was not affected by the presence of *E. parasitica* on the nuts and none of the tree stems appeared to be infected from the nut.

1981 Harvest

In September 1981, we harvested unopened burs from six American chestnut trees at Lockwood Farm. The burs were placed in a moist, cool root cellar

for a week prior to removing the nuts from the opening burs. None of the nuts were observed to be infected at that time.

Nuts of each tree were divided into three equal groups of 50 to 100 nuts and placed in plastic bags with damp peat moss. One group was kept in the root cellar (about 13 C), another placed in a refrigerated room (about 4 C), and the third kept at room temperature (about 21 C).

The nuts were examined after 11 weeks for the presence of *E. parasitica*. Some nuts from each of the six trees had apparent *E. parasitica* infections (Table 1). Nuts that were stored at room temperature showed the most infection (i.e., mycelial fans or pustules). None of the nuts stored at the cooler temperatures produced fruiting bodies that erupted through the shell. More nuts from the 1981 harvest will be examined and *E. parasitica*-like isolates characterized.

Table 1. Number of nuts apparently infected by *E. parasitica* over total stored at three different temperatures, 1981.

	Approximate storage temperature - 11 weeks		
Tree	21 C	13 C	4 C
1	40/100	0/100	0/100
2	57/100	4/100	0/100
3	23/100	0/100	3/100
4	77/100	7/100	12/100
5	63/75	3/75	4/83
6	44/50	9/50	1/42
Total	304(57.9%)[a/]	23(4.4%)	20(3.8%)

[a/] Fruiting bodies erupted through the shell of 54 of the 304 (18 percent) infected nuts.

Discussion

The reports by Collins and our original observations of infected nuts do not preclude the possibility that infection occurs on the ground after release from the bur. Our more recent results suggest, however, that a high proportion of the nuts become infected while still on the tree and enclosed within the bur. A period of incubation is necessary before such infections produce the typical orange mycelial fan. There was no consistency as to where the infection appeared on the nuts, occurring at the stylar end, hilum, or in between. Some infected nuts were weeviled but many unweeviled were also infected. Therefore, oviposition does not seem to be responsible for the infections. Infection by germination of *Endothia* spores on the style and subsequent growth into the shell is a possibility.

Conclusions

Whether nuts infected with *E. parasitica* have any role in the dissemination of hypovirulent strains was not conclusively determined. It is clear, however, that nuts apparently free of *E. parasitica* at harvest may have latent infections in the shell which would not be killed by a surface sterilant. It is important that infected nuts not be exported to blight-free areas such as the western United States, Argentina, New Zealand, or Australia where blight susceptible chestnut trees are now being grown.

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EXPERIMENTATION WITH HYPOVIRULENT *ENDOTHIA PARASITICA* IN MICHIGAN

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ABSTRACT.--*Samples of Endothia parasitica obtained from American chestnut trees in Michigan have been isolated in pure culture according to standard techniques. Isolates from virulent populations and presumed hypovirulent populations of Michigan E. parasitica are presently in culture. Michigan virulent strains of E. parasitica are normal in appearance and cause cankers on excised chestnut wood that are similar in size to those reported elsewhere. Michigan American chestnut trees with healing or quiescent cankers have been tested for susceptibility to virulent forms of E. parasitica. The response of these Michigan trees suggests that they are not resistant to disease. Strains of E. parasitica from healing stands appear morphologically normal or abnormal in culture. These strains of E. parasitica possess dsRNA that is capable of being transferred to vegetatively compatible virulent strains of E. parasitica in culture and on excised dormant chestnut wood. Field tests were initiated in the spring of 1981 to determine the possibility of using native Michigan strains of hypovirulent E. parasitica to control virulent populations of E. parasitica in Michigan. The results of laboratory tests and field trials are presented.*

Most of the *Castanea dentata* occurring in Michigan has been established outside its natural range by early orchard fruit growers. There are 600 to 800 individuals 61 cm to 152 cm diameter and thousands more if smaller trees are taken into account. *Castanea dentata* occurs primarily in the west and northwestern portions of the lower peninsula in groves with 7 to 3,000 trees. The distribution of *C. dentata* is not continuous and the groves can be regarded as islands semi-isolated from each other in terms of reproduction and in terms of the strains of *Endothia parasitica* they may harbor.

In relation to *E. parasitica, C. dentata* exists in three situations: (1) *C. dentata* that remain free of *E. parasitica* and resultant disease; (2) *C. dentata* infected with *E. parasitica* that causes "normal" disease symptoms; and (3) *C. dentata* infected with strains of *E. parasitica* causing abnormal, healing cankers.

Endothia parasitica has been present in Michigan since the late 1920's and has gradually been spreading across the state. Presently, few groves remain free of *E. parasitica*. We are of the opinion that those few stands of *C. dentata* remaining free of chestnut blight have simply avoided the disease. We consider some stands to harbor virulent forms of *E. parasitica* because the disease spreads rapidly and causes complete destruction of the periderm and secondary phloem with resultant adventitious shoots developing below the girdling canker. Numerous stromata protrude from the diseased bark producing pycnidia and perithecia and eventual death of the infected tree occurs.

Eight of the 10 stands of *C. dentata* we have visited have two types of abnormal, healing cankers. The first type was initially normal in that it caused complete destruction of the periderm and secondary phloem. The remains of old mycelial fans can be seen on the surface of the secondary xylem. At the same stage of canker development all further growth of the canker ceased and the tree began healing over the quiescent canker (Figure 1A). The second type of abnormal canker is completely superficial (Figure 1B).

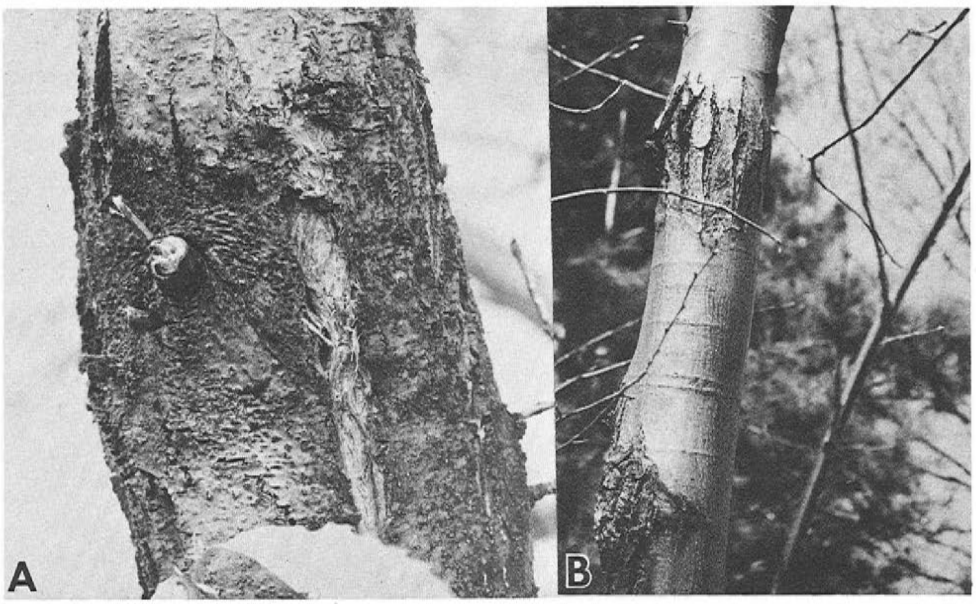


Figure 1. Types of healing cankers in Michigan. A. Healing canker that was initially expanding but now appears in remission with new chestnut tissue encroaching over the margins of the canker. B. Superficial canker completely encircling the stem.

A superficial canker may completely encircle a stem but girdling does not occur. Adventitious roots do not develop below the canker. Hand sections have revealed that the mycelium is confined to the periderm and does not produce broad mycelial fans. Stromata are rare and we have observed no perithecia or pycnidia. Both types of abnormal cankers appear to be similar to healing cankers on *C. sativa* in Italy. Healing cankers in Michigan appear to lead to a gradual improvement in the health of individual trees and entire stands.

A stand of *C. dentata* in Grand Haven, Michigan, is an excellent example of trees with healing cankers. According to the property owner, chestnut blight was apparent in the stand by 1945 and initially caused severe damage. Presently the disease is almost completely in remission with little yearly dieback. The property owner maintains that the health of the trees has improved over the last 15 to 20 years. To check the unlikely possibility that these trees may have resistance to *E. parasitica*, we removed dormant branch wood from three individuals and tested it in the laboratory with four virulent strains of *E. parasitica* and one strain from Grand Haven. Table 1 shows the sizes of developed cankers after 5 weeks incubation. The developed cankers

Table 1. Canker development after five weeks incubation on excised Grand Haven dormant chestnut wood.

Endothia parasitica strain number	Canker size
	mm^2
69	1591
59	1093
89	1752
CL4	1226
351	1420
GH2	787

are smaller than those reported elsewhere, but probably do not indicate that these trees are resistant. The excised branches though small (approximately 10 cm diameter) were quite old and had a well-developed rhytidome as opposed to a simple periderm found in younger branches of similar size. A more mature bark may slow the tangential spread of *E. parasitica*. Young excised wood from other stands of *C. dentata* with healing cankers showed more normal response to virulent *E. parasitica* (Table 2).

Sixteen isolates of *E. parasitica* obtained from bark samples collected at Grand Haven were established in pure culture as mass isolates in Difco potato dextrose agar (PDA). In culture, these Grand Haven strains more clearly resembled typical virulent strains in terms of growth rate, pigmentation and sporulation. The virulent strains used for comparison are a group from Eastern North America supplied to us by Anagnostakis and a group isolated from a virulent infection at Crystal Lake, Frankfort, Michigan.

Pathogenicity tests were performed using these 16 strains of Grand Haven *E. parasitica* on excised dormant chestnut wood from a single coppice group. These 16 strains represent a large sample of *E. parasitica* population at Grand Haven. Seven virulent strains were used as a control. The results are presented in Table 2. There is considerable variation in canker size. The size of cankers from some Grand Haven isolates overlapped with the size of cankers produced by the virulent strains, however, when the canker sizes produced by Grand Haven *E. parasitica* are averaged and then compared with the average for the virulent strains, the average canker produced by the

Grand Haven *E. parasitica* population is approximately one-third of the average canker produced by the tested virulent strains.

Table 2. Canker development by Grand Haven strains of *Endothia parasitica* compared to "virulent" *E. parasitica* on dormant chestnut wood. The same wood was used for each strain.

"Virulent"		Grand Haven	
E. parasitica strain number	Canker size	*E. parasitica* strain number	Canker size
	mm^2		mm^2
351	7200	2	571
59	2858	4	595
69	1440	5	1344
89	4468	6	1747
CL1	4333	7	2371
CL2	4733	8	1196
CL4	2707	U1	2176
		U2	1440
Average	3963	U3	986
		U4	96
		12	880
		1B	2240
		A	1500
		14	1240
		17	121
		3	1815
		Average	1270

We suspected that this reduction in capacity for disease by the Grand Haven *E. parasitica* may be due to transmissible hypovirulence. We tested Grand Haven strain 2 (GH2) and Grand Haven strain U4 (GHU4) for the presence of dsRNA. The GHU4 is an abnormal strain morphologically in culture and is nearly avirulent (Table 3). The GH2 appears normal in culture and produces a somewhat larger canker on excised wood (Table 3). Both of these strains have dsRNA as determined by polyacrylamide gel electrophoresis.

Table 3. Canker development by CL1, GHU4, and converted CL1 on dormant chestnut wood.

Endothia parasitica strain	Canker size	Average
	mm^2	mm^2
CL1	2655, 3500, 2160	2772
GHU4	100, 64, 0, 49, 64, 96	62
Converted CL1	0, 0, 51	17

Grand Haven U4 converts the morphological and pathogenic characteristics of Crystal Lake strain 1 (CL1) (Table 3). This conversion is correlated with the transfer of dsRNA from GHU4 into CL1 and occurs 100 percent of the time on PDA and in excised chestnut wood. The CL1 and GHU4 appears to be vegetatively compatible thus accounting for the 100 percent conversion frequency. The GHU4 converts the morphological and pathological characteristics of CL4 approximately 30 percent of the time on PDA. In the other 70 percent, a barrage indicative of an incompatible reaction is produced between the strains when paired on PDA. Conversion may not be the result of a complete and long lasting anastomosis between two vegetatively compatible strains. When CL4 is converted by GHU4, no barrage is produced. Genetic incompatibility lack between GHU4 and CL4 is not a barrier to the transfer of dsRNA since GHU4 converts CL4 most of the time on excised dormant chestnut branches.

Crystal Lake 1 and CL4 were isolated from a large grove of 3,000+ *C. dentata* (3,000 or more trees) at Crystal Lake, Frankfort, Michigan. This stand is presently infected by a virulent population of *E. parasitica*. Since in the laboratory we were able to convert CL1 and CL4 with GHU4, we decided to initiate a field experiment to determine if we could control CL1 and CL4 with our stock of "compatible" hypovirulent strains. The CL1 and CL4 were used to establish multiple cankers on disease free saplings 13 to 18 cm diameter by using the cork borer technique. The cankers were allowed to develop for 5 weeks and were challenged by one of four hypovirulent strains. Figure 2 shows a conversion and/or vegetative compatibility matrix between the Crystal Lake virulent strains and the Grand Haven hypovirulent strain. The GHU4, GH2, converted CL1 and converted CL4 were used to challenge the established CL1 and CL4 cankers. Single virulent cankers were established to replicate each challenge 12 to 15 times. The CL1 and CL4 cankers were allowed to develop for 5 weeks before challenging and placing mycelia from the appropriate hypovirulent strain into four cork bore holes around each canker. The challenges to CL1 cankers were started on June 4, 1981 and the challenges to the CL4 cankers were started on July 2, 1981. Measurements of the CL1 and CL4 cankers were made prior to challenging. Four bore holes were also placed around the control canker but no hypovirulent inoculum was placed in the holes.

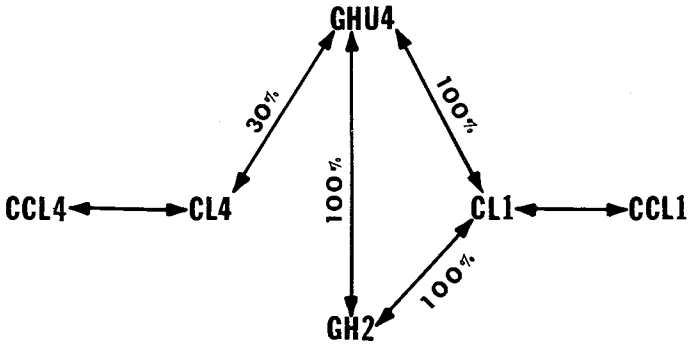


Figure 2. Compatibility and/or conversion matrix between two Grand Haven hypovirulent strains and two Crystal Lake virulent strains.

In mid-October, the cankers were measured to determine the difference in size, if any, among the cankers challenged with the four hypovirulent strains, compared to the control cankers. Figure 3 presents this data for the CL1 control and the hypovirulent challenges to CL1. The data for CL4 are similar.

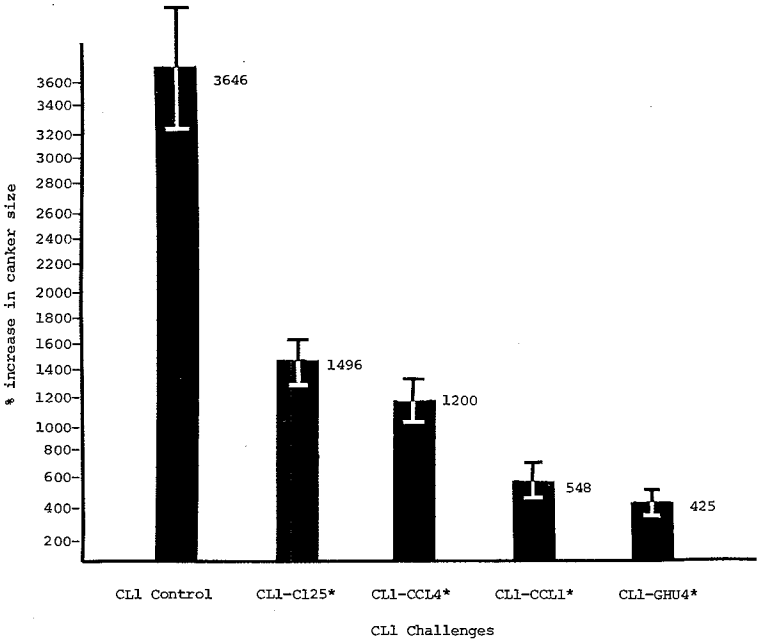


Figure 3. Percent increase in growth of control CL1 cankers and the challenge CL1 cankers during the summer of 1981. Bars equal one standard error of the mean challenging strains.

but this experiment has not run long enough for us to be certain of the results. The bar graph represents the percent increase in canker size (mm^2) from the time of challenge to the final canker measurement in October. During this time interval, the size of the unchallenged CL1 control cankers increased by an average of 3,646 percent whereas the cankers challenged with the hypovirulent strain GHU4 converted CL1 and converted CL4 increased significantly less. Some size increase with the challenged cankers was expected because the hypovirulent strains caused a canker of their own around each of the four bore holes. Some CL1 cankers were challenged with *E. parasitica* strain CL25 isolated from a canker at the Crystal Lake grove. The small percentage of canker size increase is probably due to inhibition or competition between these two strains. Crystal Lake 25 does not possess dsRNA. The differences in size increase between the control canker and the cankers challenged with our native hypovirulent strains should increase dramatically over time if a conversion has taken place on the trees.

We were not content to wait a couple of years to see if we could just control these small cankers we initiated. Presently, about 20 percent of the trees in the Crystal Lake grove have at least one expanding canker. We

decided to challenge 36 of the large existing cankers with our four new hypovirulent strains of *E. parasitica*. Each canker was challenged with a single hypovirulent strain by ringing the margin of the canker with cork borer holes. The borer holes were filled with the appropriate mycelia and PDA and were covered with masking tape. These challenges were initiated on June 4, 1981. At the same time, we collected bark samples from each canker and established pure cultures of *E. parasitica* from each canker. Every isolate appeared normal in culture. We will use these cultures as references for what *E. parasitica* strain was causing the canker. Later if the challenged cankers go into remission, we can sample for possible conversion and transfer of dsRNA. Shortly after establishing the challenge to the cankers, we outlined the margin of other existing cankers that we did not challenge. This was done so that we could compare the growth of a normal canker on these medium sized trees.

Thirty-three of the 36 challenged cankers ceased expanding for the growing season. Healthy new host tissue was present at the margins of the same challenged cankers. These cankers resembled the early stages of healing cankers elsewhere in Michigan that were initially virulent and became converted naturally. The new tissue at the margins of the cankers appeared free of *E. parasitica*. We sampled the margins and centers of these cankers for *E. parasitica* and were able to obtain some cultures that were morphologically abnormal in culture as are the hypovirulent strains used for the challenge.

All the hypovirulent challenging strains that we used produced very small cankers (under 100 mm^2) when inoculated alone in *C. dentata*. These strains may control individual cankers but they may be too debilitated as pathogens to persist, spread and establish additional hypovirulent cankers and hypovirulent inoculum. In mid-summer, we found dsRNA in GH2. The GH2 produces a persistent long-lived canker on *C. dentata* in the Grand Haven grove and produces a relatively small canker with spore horns on excised dormant *C. dentata* wood. We are presently evaluating the ratio of normal to hypovirulent conidia produced by GH2. Grand Haven 2 strain was used to challenge a large established Crystal Lake canker on July 26, 1981. The GH2 may be an appropriate native hypovirulent strain to establish at Crystal Lake because it produces a long-lived persistent superficial canker at Grand Haven. Such a hypovirulent canker could be an important source of hypovirulent inoculum. The somewhat increased virulence of GH2 seems to be within the tolerance of *C. dentata* in Michigan.

THE DISTRIBUTION OF SURVIVING AMERICAN CHESTNUTS IN MICHIGAN

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ABSTRACT.--*Two-hundred-twenty-four locations of American chestnuts were examined in Michigan between 1975 and 1981. Eighty-four of these locations were diseased while 140 sites were disease-free. Of the 1094 trees recorded with diameters greater than 15 cm d.b.h., a total of 645 were diseased or occurred at diseased sites whereas 349 were located at sites where blight was not observed. Most trees at locations where blight occurs suffered extreme damage during the first few years of infection. However, 24 locations in southwestern Michigan and 5 locations in the Northeast contain trees where superficial cankers are common 10 to 20 years after the disease was first observed.*

In 1975, I began a study on the American chestnut in Michigan. The native range of *Castanea dentata* extended into southeastern Michigan (Saucier 1973), but early settlers planted the tree beyond the native range. The objective of my research was to locate planted or native American chestnuts and record number of trees, heights, circumferences, and condition with respect to chestnut blight. When possible an estimate of the time blight was first observed in the area was obtained from nearby residents.

Over the last 6 years locations from herbarium specimens were gathered and letters sent to botanists, foresters, county agents, and other knowledgeable individuals. When there was doubt as to whether a tree was an American chestnut, Graves (1961) was used for species identification. Voucher specimens from each location were placed in the Clarence Hanes Herbarium at Western Michigan University. Slides taken at each site are in the author's possession.

Results

Two hundred twenty-four locations were examined during the study (Figure 1). Eighty-four of these locations were in various states of disease while 140 were disease-free. Of all the trees with diameters 15 cm or greater, 645 were diseased or occurred at diseased sites, whereas 349 were found at sites where blight was not observed. Several locations in the upper part of the lower Peninsula have 15 or more large blight-free trees. One of the most impressive sites is in Missaukee County where there are 27 blight-free trees 25 cm d.b.h. or greater with 2,500 naturally produced saplings or small trees 15 cm d.b.h. or less. Only 42 blight-free trees were found

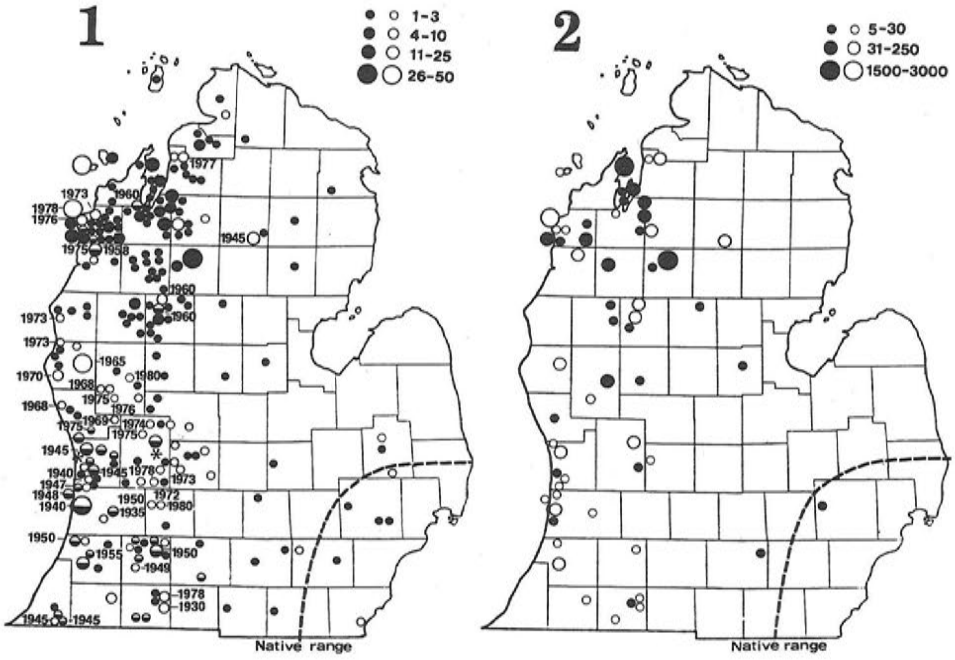


Figure 1. Distribution and number of American chestnuts in Michigan greater than 15 cm d.b.h. Completely dark circles indicate blight-free locations while the open circles represent diseased sites. The half dark circles are diseased locations that show signs of surviving the blight. The two sites with asterisks have confirmed hypovirulent strains. The dates are the approximate times when the blight entered specific locations as conveyed by nearby residents. The size of circles indicate average stand diameter,d.b.h., in inches.

Figure 2. Distribution and number of naturally reproduced American chestnuts in Michigan. Dark circles indicate blight-free locations; open circles represent diseased sites. The size of circles indicate relative numbers of trees at each site.

in the lower half of the Lower Peninsula; no blight-free groves were found. Only two American chestnut locations were reported from the Upper Peninsula.

The distribution of many of these chestnuts appears to be correlated with old homesteads and orchards. With the exception of the Manistee National Forest, the fruit belt along the western portion of the Lower Peninsula provided the greatest concentration of trees.

In this study, few American chestnuts were found in the native range. This was due partly to the development around the Detroit area and partly because the tree was never very abundant in its Michigan native range. An examination of the original land surveys of 80 townships in southeastern Michigan revealed only five American chestnuts recorded as witness trees. This suggests to me that the tree composed less than 1 percent of the original forest in its native range in Michigan. However, Davis (1976) has pointed out through pollen studies that *Castanea dentata* moved into Michigan less than 1,000 years ago and that at the time of the blight the chestnut was still migrating. Data from this study supports the idea that the American chestnut

was migrating northward and would have done well in other parts of Michigan. Figure 2 shows the locations of small trees and saplings under 15 cm d.b.h. which have reproduced naturally. In addition to the Missaukee County site mentioned above, one location in Leelanaw County also has 1500 impressive small trees and saplings. If these two populations are not infected in the next 50 years, they should become small chestnut forests. The stand described by Thompson (1967) had an estimated 3000 naturally produced chestnuts 15 cm d.b.h. or less in 1978 when the blight was first thought to have entered.

How far north the American chestnut would have migrated in Michigan is debatable. When planted, the tree is able to grow and reproduce naturally throughout the lower peninsula. However, the fact that damage was done to trees in the cold northeastern part of the Lower Peninsula during the winters of 1977-78 and 1978-79 indicates that the American chestnut probably would not have done well in that part of the state. The two locations reported from the upper peninsula (not visited) are probably sheltered from extreme cold or great fluctuation in temperature because of their proximity to Lake Superior.

The examination of the diseased trees proved to be the most interesting aspect of this study. The earliest record of blight in Michigan was a grove in St. Joseph County in which blight was described in 1930. Although these trees have been stripped of their bark for over 50 years, some of them are still standing. Most of the trees in the native range were probably blighted in the 1930's and 40's also. As a result many of these trees have been removed. Most of the large groves in southwestern Michigan were diseased in the 1940's and early 50's. The majority of the diseased locations in the northwestern part of Michigan were infected in the last 10 years.

From the start it was obvious that some trees in Michigan were more damaged by blight than others (Figures 3 and 4). However, it was not known whether some trees were more resistant or certain strains of the fungus more deadly. In 1977, after reading the article by Van Alfen et al. (1975) and talking with the individuals from the Connecticut Agricultural Experimental Station, it became obvious that the situation in Michigan was in some ways similar to that in Europe where Michigan American chestnut trees have cankers that have callused (healed) naturally.

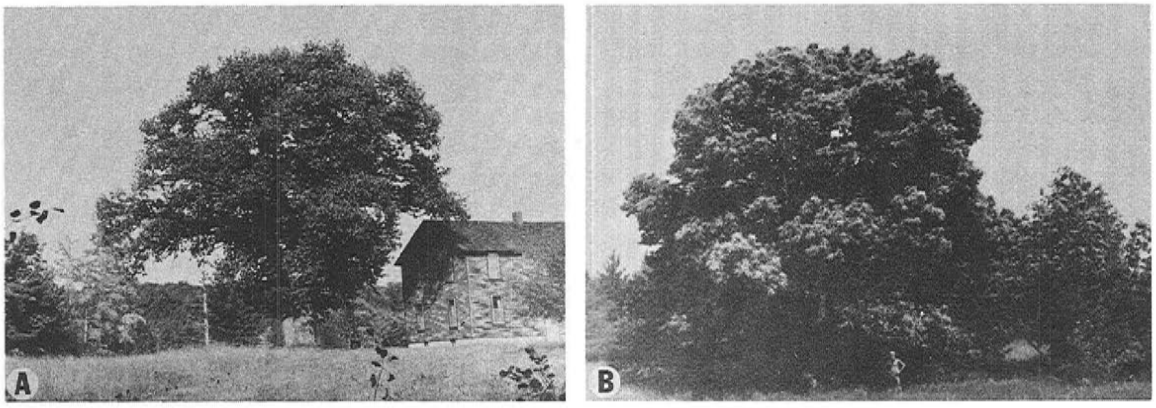


Figure 3. A. A large blight-free American chestnut (168 cm d.b.h.) in Lake County, B. This American chestnut tree was from Kalkaska County.

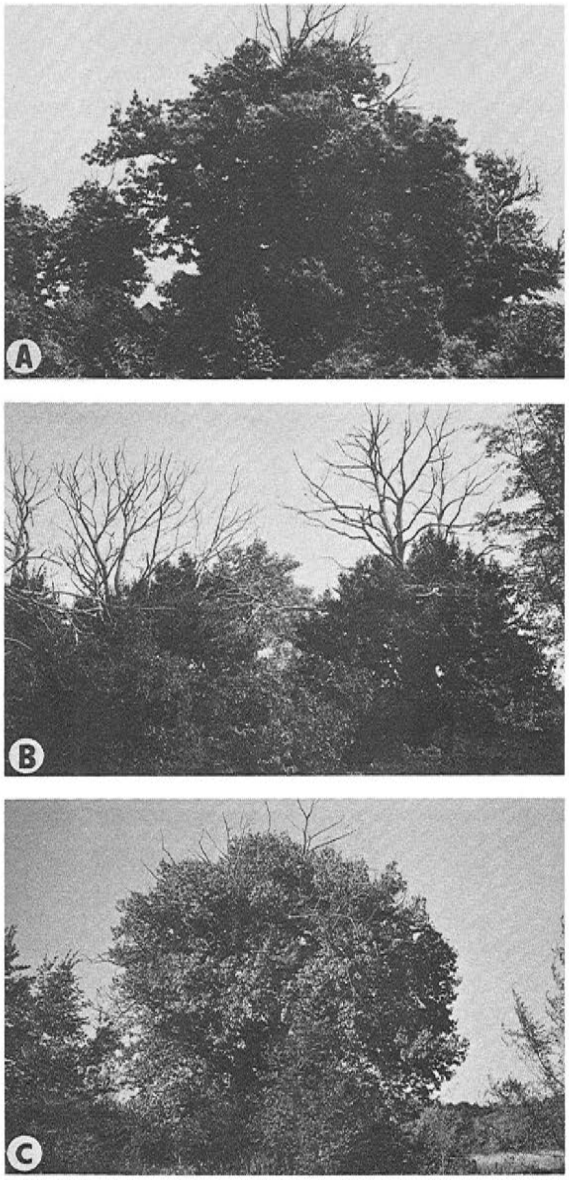


Figure 4. A. Mary Reinoldt reported American chestnuts near Grand Haven in 1975; trees have had the blight for over 35 years and possess hypovirulent strains of *Endothia parasitica*. B. Chestnut trees were reported by Thomas Reuschal and have had the blight for over 23 years (middle photo). C. Richard Pippen reported this tree (bottom photo) near Bangor in 1975; this tree has had the blight for over 25 years and has many superficial cankers.

The first record of hypovirulence in Michigan and America came from a grove near Rockford, Michigan, in 1976 when Priscilla Johnson sent bark samples to the Connecticut Agricultural Experimental Station. They proved to have double-stranded RNA characteristics of hypovirulent strains and were able to cure cankers incited by virulent strains (Anagnostakis 1978). In 1978 hypovirulent strains were also confirmed from a site near Grand Haven, Michigan by Peter Day. The Grand Haven location was reported to me by Mary Reinoldt in 1975. According to George Unger, who has lived on the Grand

Haven site more than 70 years, the blight entered the site about 1945 at which time the trees began to die rapidly. However, after about 10 to 15 years the death rate slowed so that new growth began to equal the amount of dieback. In recent years only small branches have died (Figure 4). Cores taken from a number of larger trees show that most trees have maintained a slow rate of growth in the last 30 years with one tree showing a dramatic increase in growth during the period. The smaller 25- to 35-year-old trees that never suffered damage from the virulent strains have maintained a diameter growth of about 1.25 cm per year. Because of its potential value to science, this Grand Haven site is being purchased by the Michigan Nature Conservancy.

Although only the Grand Haven and Rockford sites have confirmed hypovirulence at this point, I have observed significant numbers of superficial cankers at 24 localities in southwestern and five in northwestern Michigan (Figure 1). These superficial cankers which persist for many years are nearly always swollen and show signs of continuous growth whereas the virulent cankers are nearly always flush with little or no swelling to surrounding bark, and without noticeable callusing tissue. The superficial cankers vary in shape and form from one location to another. In a couple of sites in the northwestern part of the Lower Peninsula the superficial cankers are less swollen and lack the yellowish-orange color. These cankers tend to have a callus layer on the outer portion of the bark (Figure 5).

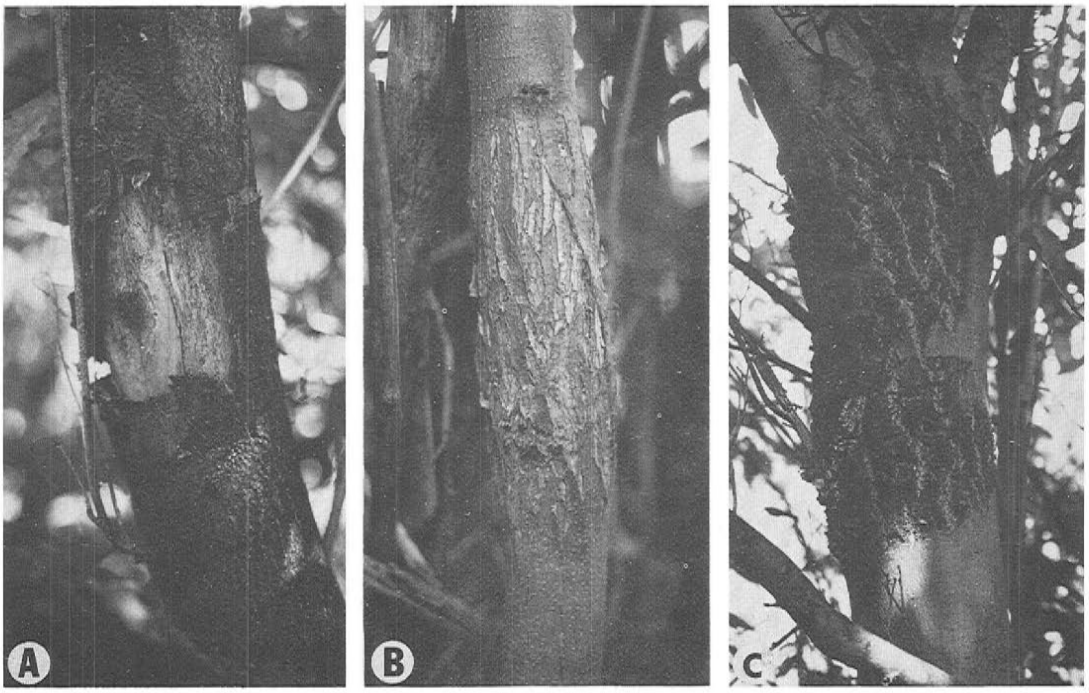


Figure 5. A. A typical virulent or killing canker taken in Allegan County. B. A swollen superficial canker from the Grand Haven location. C. A superficial canker from the Benzie-Manistee County line site. This canker is less swollen, lacks the yellowish-orange color, and has a callus layer on the outer portion of the bark.

The important fact about the Michigan trees is that in certain groves the superficial cankers have spread throughout the population and the trees have survived even though infected. The ability of trees with these cankers to survive for extended periods of time is evident from the dates in which the blight first entered, the present stem sizes, the lack of dieback, and tree cores taken at specific sites. To what extent hypovirulent strains suppress virulent strains in Michigan is not known. The fact that hypovirulent strains are not spread by wind-dispersed ascospores makes it hard to believe that they are capable of keeping the virulent strains in check. However, as Mittempergher (1978) pointed out, a possible explanation for the spread of hypovirulent strains is that they may be more adaptable in the saprophytic phase than virulent strains. If this is so, we may have areas in Michigan saturated with hypovirulent strains with very few virulent strains remaining. It may be necessary to have a reservoir of hypovirulent strains in a stand before it can be sufficiently protected from virulent strains. I have noticed that in one location near Holland, Michigan where the majority of the larger trees with superficial cankers were removed, the remaining trees have since become badly blighted. In addition, the smaller trees and saplings 0.25 mile (0.40 km) south of the larger trees at the Grand Haven location are in much worse condition than those in the middle of the grove. More research should be done to determine what density of hypovirulence is needed to protect a stand. Also, Michigan's environment may be more favorable than other states for hypovirulent strains. Consequently, further research should determine the environmental factors important for survival of hypovirulent and virulent strains.

Understanding how hypovirulent strains have been dispersed in Michigan may have significance in determining whether or not the strains can be used in biological control. The question of whether hypovirulent strains are being dispersed between groves miles apart or have arisen at various locations should be answered. Of the over 80 different blighted locations which I have observed, nearly every location has had extensive initial dieback indicating that the first strains to enter a site are virulent. It is only after the virulent strains have come in and caused considerable damage to the trees do we see signs of the swollen superficial cankers. In most cases there has been a 10 to 20 year period before the superficial cankers appear. This, I think, adds support to the idea that hypovirulence is being developed simultaneously at different sites around Michigan.

Conclusion

Although the American chestnut is very close to extinction in its Michigan native range, there are numerous locations where blight-free trees survive outside the native range. How long these trees will remain isolated from the blight will probably depend on the ability of the fungus to disperse and live on various substrates. In the last three years over 150,000 American chestnut seeds collected by James R. Comp, Sr., and others in the Cadillac area have been planted by the Soil Conservation Department. Although these trees may provide new host material for the blight, they will probably assure the existence of at least some blight-free trees.

Since blight-free trees will always be susceptible to virulent strains of the blight the most impressive American chestnuts in Michigan are not the large blight-free trees but those which are surviving with the blight. The

fact that there are groves which suffered extensive damage earlier but are now improving and reaching mature tree size again is encouraging. The Michigan trees provide an interesting opportunity for further study. The fact that different groves of chestnuts in Michigan are separated provides for isolated populations of *Endothia*. These populations should be studied before the newly planted saplings become new host material and eliminate the barriers between them.

Whether problems such as vegetative incompatibility and dispersal can be worked out remains to be seen. However, the present status of the American chestnut in Michigan provides new hope for its survival as a tree species.

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DETECTION AND EVALUATION OF HYPOVIRULENCE IN AND RESISTANCE TO ENDOTHIA PARASITICA IN SURVIVING AMERICAN CHESTNUTS AND ASSOCIATED OAKS IN NORTH CAROLINA

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ABSTRACT.--*Cultural hypovirulence in Endothia parasitica from four American chestnuts (d.b.h. 15.6 to 33.0 cm), two each in Iredell and Alexander Counties is reported. Plans for further evaluation of hypovirulence and/or resistance in these and additional candidate trees, as found, are described.*

The search for resistance in large American chestnut, *Castanea dentata*, survivors of the epidemic caused by *Endothia parasitica* now has an added dimension with recognition that some strains of the pathogen are in themselves diseased or, by Elliston's (1981) new terminology, cytoplasmically hypovirulent (CH), as probably caused by virus-like agents (Day et al. 1977). Theoretically, the agent(s) in CH strains can be transmitted via hyphal fusion with virulent (V) strains in normally lethal cankers and reduce pathogenicity to the extent that bio-control and tree survival are attained. Transmission between CH and V strains is governed, however, by their vegetative compatibility (v-c) of which there are 77 known types in North America (Anagnostakis and Waggoner 1981). Despite this low probability for compatible CH and V strains to pair in nature, some chestnuts have survived either by means of CH in the pathogen, resistance of the host, possibly both, or disease escape. At least 20 large surviving American chestnuts in seven states have been shown to yield abnormal (potentially CH) isolates of *E. parasitica* (Jaynes 1981). Such trees need to be assessed for the possible interaction of CH and resistance to their survival.

Candidate trees for this type of study are not likely to be found by conventional survey methods. For example, our systematic survey to determine the incidence of *E. parasitica* on oaks and chestnut in the mountain and Piedmont counties of North Carolina (Nash and Stambaugh this proceedings) did not detect a single chestnut larger than 6.8 cm d.b.h. nor did the isolates from 247 trees of all species sampled show any cultural abnormality or potential CH. Quite by chance, however, in November 1980, we were apprised of a 28 cm American chestnut near Union Grove in Iredell County found by a local lumber mill buyer. Since then, the owner of the property has reserved the tree for our use and has led us to three more study trees within a 4.8 km radius of the first tree (Table 1).

Table 1. Data summary from surviving American chestnuts in Iredell (IR) and Alexander (AX) Counties, North Carolina

Tree number	D.B.H.	Number stems	Cankers height	Tissue samples	Number of isolates[a/] T	SUBC	AB
	cm		m				
IR/1	28.4	18	0 - 11.0	260	127	20	2
IR/2	33.0	14	0 - 7.9	168	140	118	12
AX/1	15.6	7	0 - 7.3	73	61	55	8
AX/2	24.6	5	0.9 - 7.0	49	46	35	13

[a/] T = total isolates obtained; SUBC = growth observed through two to four subcultures; and AB = culture abnormal, i.e. morphologically hypovirulent.

This paper reports our preliminary findings from these trees and outlines our plans to enlist the aid of some 148 hardwood lumber mill operators and buyers (NC Forest Service 1979), and through them, the logging contractors, by asking all individuals thus contacted to report the locations of surviving chestnuts, as found in the 26 counties where chestnut is known to occur (Buttrick 1925; Saucier 1973). From this solicitation and response, we anticipate detection of a sufficient number of additional trees to meet our study needs.

Materials and Methods

Candidate trees will be grouped by localities, as reported, to facilitate ground-scouting. During the first visit, a candidate tree will become a study tree if it meets a diameter minimum of 21.6 cm and is either canker-free or displays sublethal infection. Determination of ownership and permission to sample will precede the second visit and will be scheduled during tree dormancy so that at least 20 scionwood cuttings can be taken simultaneously with procurement of 10 to 20 bark core samples per canker perimeter. Isolates from this material will be screened for within-tree v-c type frequency and distribution by the methods of Anagnostakis (1978) and for the appearance of cultural CH by observation of colony growth through four 9 cm plate subcultures; those isolates expressing cultural CH will be tested against within-tree normal V isolates for conversion capacity, using the methodology of Anagnostakis and Day (1979). Their pathogenicity will be compared with V isolates using paired inoculations of living sprout stems of American chestnut. If CH is indicated by preliminary laboratory findings, the source tree will be revisited to determine whether CH strains have spread from this locus by sampling all trees symptomatic of *Endothia* infection within cardinal-direction transect radii of 152 m. The isolate yields from this population sample will be screened in the laboratory for cultural CH detection and intra-tree v-c type distribution as compared with those present in the source tree.

All scionwood material will be stored at 5 C until March when side grafts will be made to Chinese chestnut, *C. mollissima*, rootstock already planted on two sites in the Duke Forest. Once established, the scions will be inoculated by standardized procedure to determine whether the source tree actually possesses some degree of resistance.

Results

Symptomatology. Tree data given in Table 1 actually represent only three locations since AX/1 and 2 were only 6 m apart. All trees displayed full, healthy crowns with cankering confined to main stems. Canker distribution ranged from ground level to the first few live branches at 7 to 11 m (except for AX/2). Basal cankers were deep seated with pronounced marginal callus. Tree IR/1, the first to be discovered, had the most distinctive and uniform symptoms expressed as roughened, slightly fusiform-shaped swellings (Figure 1). *Endothia parasitica*, in relation to these symptoms, was superficial in the outer bark and necrosis, if any, was shallow. The lowermost canker in tree AX/2 was also unusual in that it extended continuously from 0.9 to 3.6 m and appeared as exposed, roughened inner bark with a definite orange cast. All other cankers were atypical of chestnut infection in that callusing was quite evident.

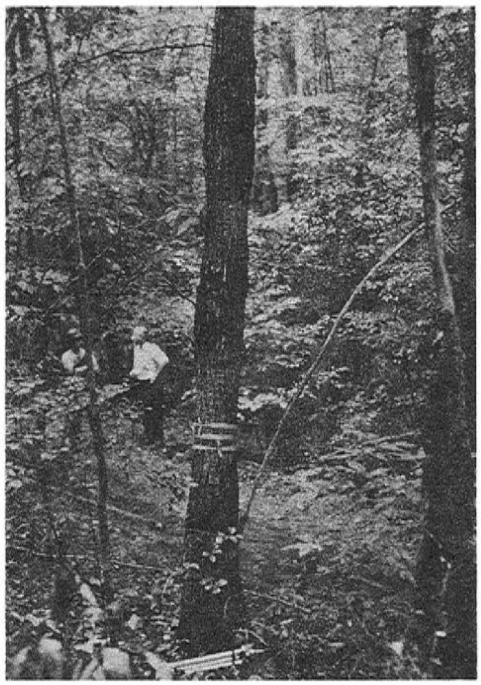


Figure 1. Lower stem of a surviving, 28.5 cm d.b.h. American chestnut in Iredell County, North Carolina showing fusiform swellings associated with *E. parasitica* infection.

Isolate Yield and Characterization. Cankers on all trees were sampled to a height of about 6 m, i.e. within the safe-use limits of our Swedish climbing ladder. Isolation yield of *E. parasitica* from all canker-perimeter, bark-core samples taken per tree is shown in Table 1. Yields ranged in frequency from 48 to 94 percent, as represented by IR/1 and AX/2, respectively.

Pairing of all within-tree isolates among themselves to determine the number of v-c types is nearing completion with v-c type maxima relative to total isolates (Table 1) reduced to 14 (IR/1), 80 (IR/2), 41 (AX/1), and 38 (AX/2). Observations on colony growth of 20 or more isolates per tree through two to four subcultures, to date (Table 1), has revealed at least 2 to 13 abnormal strains from each, as based on reduced growth, lack of pigment, and irregular colony margin, singly or in combination. An additional 60 isolates were obtained from eight scarlet oaks, *Quercus coccinea*, one white oak, *Q. alba*, and an additional 12.7 cm d.b.h. chestnut, all within 365 m of AX/1-2; none of these isolates have shown cultural abnormalities as yet.

Scionwood Grafts. Thirty-one branch tip cuttings from IR/1 were side grafted to Chinese chestnut rootstock on April 2 and 3, 1981; by June, 58 percent of these attempts were judged successful but this dropped to 13 percent by fall. Scionwood from other sources, namely an 82 cm American chestnut on Carter Mountain in Wilkes County and a susceptible American chestnut sprout in Watauga County, gave slightly better grafting results. Survival this fall, was 3/14 (22 percent) and 5/15 (33 percent) respectively.

Discussion

Our results at this stage are primarily descriptive of symptoms in four large surviving American chestnut trees and suggestive that cytoplasmic hypovirulence in the pathogen may be contributing to their survival.

Certainly, symptomatology as described and particularly as illustrated in Figure 1 is atypical of cankering caused by virulent strains of *E. parasitica*. The superficial position of the fungus in stem swellings and the pronounced callus formation in most other cankers is taken as evidence that either resistance, cytoplasmic hypovirulence, or both are functioning.

Our isolation results from each of the study trees (Table 1) indicated detection of some culturally abnormal isolates of the pathogen. These were usually expressed as drastically reduced growth rates on potato dextrose agar in plate culture and deeply lobed colony margins. We recognize that demonstration of cytoplasmic hypovirulence in these isolates will require substantiation, including: 1) conversion ability in dual culture with V-strain isolates (Anagnostakis and Day 1979) with due attention to cultural stability problems (Van Alfen et al. 1978); and 2) pathogenicity compared with that of V-strain isolates by inoculation and measurement of necrosis in living American chestnut. Our plans for the former will match potential CH-strain isolates with V-strain isolates on the basis of within-tree/canker v-c type patterns once that data is complete. For the latter, all culturally abnormal isolates accumulated by next spring and representative V-strain isolates will be inoculated on American chestnuts within a sprout stand located in Watauga County. Since isolates from the AX and IR trees have shown considerable latent expression of growth abnormality which is not easily detectable in tube culture, we are routinely following colony growth in Petri plates through four subcultures.

If CH-strain presence can be adequately demonstrated in any or all of the AX and IR trees, their accessibility at the extreme northern juncture of the two counties and their proximity to one another within an 8.1 km^2 area, should prove a valuable asset to our studies. In the search for additional study trees, four other American chestnuts in Buncombe, Caldwell, and Wilkes counties, with d.b.h.'s ranging from 29 to 82 cm, meet our specifications and will be examined pending permission from the respective owners.

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PROPAGATION OF AMERICAN CHESTNUT IN VITRO[1/]

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ABSTRACT.--*Axillary shoots developed on mature embryos of American chestnut seeds which were cultured on 6-benzylaminopurine (BAP-supplemented Murashige and Skoog (MS) medium. These shoots could be multiplied by subculture on MS medium with reduced BAP concentration. Rooting of some of these shoots occurred when they were placed on 3-indolebutyric acid (IBA)-supplemented medium followed either by culture on IBA - free medium, or by planting in vermiculite and placing them in a greenhouse mist propagation bed. Plantlets have been successfully transferred to soil in pots and grown outdoors. Axillary shoots have also been obtained from buds of 4-month-old greenhouse-grown seedlings.*

Although it has received less attention in recent years, the selection and breeding of chestnut trees has been continued by several researchers and organizations (Given and Haynes 1978; Jaynes 1978; Keys et al. 1975; Thor 1978). The goal of these programs is to produce blight-resistant, timber-type chestnuts. In order to multiply selections or hybrids for outplanting, a reliable, inexpensive, and rapid method of asexual propagation is necessary. The techniques which have been used to propagate chestnut are either inadequate or are too costly (Keys 1978).

One possible technique would be the propagation of chestnut using *in vitro* shoot culture. Plantlet formation has been reported in apple (Jones et al. 1977), almond and almond-peach hybrids (Tabachnik and Kester 1977), European alder (Hosier et al. 1981), cherry (Ivanicka and Pretova 1980), and plum (Hammerschlag 1981) using the shoot culture technique. Vieitez and Vieitez (1980) reported plantlet formation of European chestnut. Keys and Cech (1981) reported the *in vitro* plantlet formation of American chestnut using shoots of embryonic origin.

This paper describes the methodology used in the culture of embryonic shoots and seedling buds of American chestnut. Results of various attempts to improve the rooting of these shoots and establishment of the resulting plantlets will be discussed.

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Culture Initiation

Shoot cultures were initiated using excised embryos of stratified American chestnut seeds. The seeds were stratified in moist sand in plastic bags for 60 days at 1 C. Surface sterilization was accomplished by dipping the seeds in 95 percent ethanol for 1 minute, followed by flaming and cooling in sterile distilled water. The seed coat was then cut and peeled away. The embryo was excised and placed radicle-end-down on 30 ml of nutrient agar medium in a 25 x 150 mm culture tube which was then sealed with aluminum foil.

Buds of greenhouse-grown American chestnut seedlings were also used to initiate shoot cultures. Stem sections were cut and soaked overnight in distilled water, with 0.1 percent Alconox as a wetting agent. The stem sections were surface sterilized by soaking in 5 percent sodium hypochlorite (100 percent commercial bleach solution) for 10 minutes followed by three rinses in sterile distilled water. The stems were cut into 1 cm - long sections, each having one bud. Each explant was placed vertically on 50 ml of nutrient agar medium in a 125 ml Erlenmeyer flask which was then sealed with aluminum foil. Both types of cultures were grown in growth chambers at 27 $\pm$ 2 C under a 16-hr photoperiod of 200 fc of fluorescent and incandescent light. After 4 to 6 weeks, a whorl of axillary shoots developed at the base of the original explants.

The initiation medium consisted of Murashige and Skoog (MS) (1962) macro and micro nutrients supplemented with (per liter) 0.5 mg niacin, 0.5 mg pyridoxine•HCl, 0.1 mg thiamine•HCl, 100 mg myo-inositol, 30 g sucrose, and 1.0 mg 6-benzylaminopurine (BAP). The pH of the nutrient medium was adjusted to 5.5 to 5.6 with 1N NaOH prior to filtering and autoclaving. The medium was solidified with 6 g/l Phytagar (Gibco).

Shoot Multiplication

Individual shoots which were at least 1.5 cm long, or clumps of shoots, were excised from the original explant and subcultured in order to further multiply the number of shoots. The subcultures were placed on 50 ml of nutrient medium in 125 ml Erlenmeyer flasks and were grown under the same conditions as the original explants. The shoot multiplication medium was the same as the culture initiation medium except that the nitrates were reduced by half, and only 0.1 mg/l BAP was added.

New shoots formed in 4 to 6 weeks. Average shoot production was approximately 10 per culture. The ability to produce axillary shoots did not decrease, even after eight subcultures. In this way, as many as 800 to 1000 shoots per year could have been produced from each original explant.

Root Initiation

Shoots which were at least 1.5 cm long were subcultured to a root induction medium. This medium consisted of MS salts with the nitrates reduced by one-half, and supplemented with 1.0 or 3.0 mg/l of 3-indolebutyric acid (IBA). The shoots were maintained for 1 or 2 weeks on this medium in the previously described environment. After this period, the shoots were subcultured to the

same medium without hormones to allow roots to develop. Rooting occurred in 4 to 6 weeks (Figure 1). At present, the methods tested for root initiation have not produced adequate results. Rooting has occurred on only 4 percent of the shoots which were subcultured to the root induction medium. Further tests showed that concentrations of IBA above 3.0 mg/1 stimulated excessive callusing at the base of the shoots, which inhibited root development. Tests using 3-indole acetic acid (IAA) or naphthaleneacetic acid (NAA) instead of IBA failed to induce rooting.

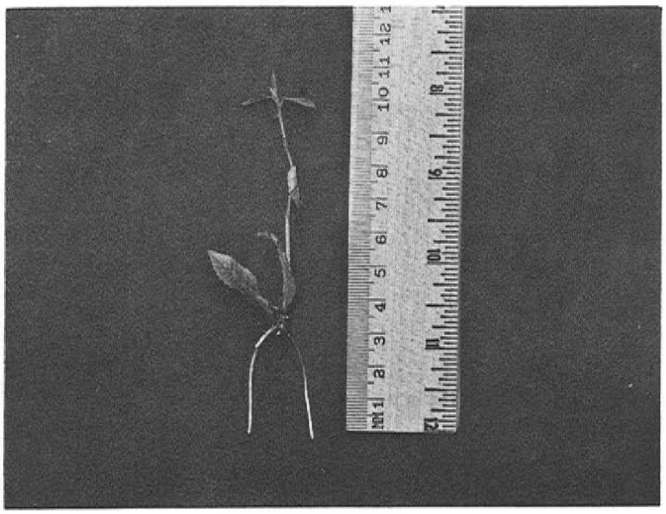


Figure 1. American chestnut plantlet produced *in vitro*.

Another technique showed slightly better preliminary results, with 17 percent (3 of 18) of the shoots rooting. One of the shoots that rooted using this technique was of seedling origin. The shoots were dipped for 1 second in 5000 mg/1 of IBA in 95 percent ethanol, followed by culture on hormone-free, reduced-nitrate medium. Basal callusing was severe on many of the shoots, indicating that the concentration of IBA might have been too high. The same technique using IAA failed to induced rooting.

Establishing Plantlets in Soil

The transfer of the rooted shoots from the sterile culture condition to soil outdoors had to be done gradually. The plantlets were removed from the medium and the roots were washed to remove any remaining agar. They were then planted in vermiculite which had been soaked with half-strength MS salts. The plantlets were placed on a greenhouse propagation bed under intermittent mist until the roots were well developed (at least 3 weeks). After this time, they were transferred to soil in pots and grown outdoors under a shade house. The root system had to be well-developed, or the plantlets died during this stage. One plantlet was successfully transferred to soil using this method. After 4 weeks in soil, it was vigorous and putting on new growth.

Conclusions

American chestnut shoots from juvenile tissue can be rapidly and easily multiplied *in vitro*. As has been the problem with chestnut in the past, a reliable method of rooting these shoots must be developed. However, since the shoots are succulent and physiologically juvenile, the possibility of developing such a rooting technique is good. Other difficulties may exist in transferring the plantlets to soil for growth under normal environmental conditions. And the technique has yet to be tested using buds from mature trees. Once these problems are overcome, shoot culture may be a good tool for the multiplication of blight-resistant or superior American chestnut or hybrid clones.

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CARPENTER ANTS AS CARRIERS OF ENDOTHIA PARASITICA

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ABSTRACT.--*The behavior of carpenter ants has been well studied, and information from these reports as well as the presence of ants on chestnut trees made these insects good candidates as carriers of Endothia parasitica.*

Carpenter ants in the genus *Camponotus* excavate galleries in wood to form homes for their colonies. The galleries are started in spots of fungal decay, deep checks, knot holes, or other defects (Friend and Carlson 1937). *Camponotus herculeanus pennsylvanicus* was commonly found in chestnut telephone poles in Connecticut, and noted that in 1936 there were still 201,182 chestnut poles in use in this State (Friend and Carlson 1937). Colonies of all four major species of *Camponotus* can be found under rocks and in the ground, but their main nesting sites are standing dead trees or poles (Sanders 1964). Also Sanders (1964) reported that eight was the largest number of trees utilized by a single colony for nesting. There were underground entrances to 147 of 150 studied nest trees, with connecting tunnels under the forest floor litter. Sanders (1964) found 12 colonies in 54 trees on a (0.57 ha) woodlot in New Jersey. The two largest colonies each had tunnels covering about (0.02 ha) in shaded areas where heavy moss and organic debris had accumulated. This would mean that tunnels ranged out about 8 or 9 meters from the nest, assuming the nest to be at the center of a tunneled circle. Harold Fowler, currently working at Rutgers University, has observed carpenter ants in open areas traveling 30 to 35 meters from their nest to aphid colonies (personal communication).

Foraging is restricted by rainfall and temperature (Fowler and Roberts 1980), and foraging intensity varies from month to month. Pricer (1908) reported that the principal food of carpenter ants is honey dew from aphids, occasionally supplemented by insect body fluids and plant juices. Recent studies by Eisner and Happ (1962) demonstrated that particles 200 and 300 µm in diameter were not ingested by carpenter ants, and that 150 µm particles were trapped in the infrabuccal filtering pockets of the ants. Particles 10 µm and 100 µm were swallowed into the crop, with 10 µm particles being more commonly swallowed. Solid particles were not pumped into the midgut. Material can be stored in the crop for extended periods of time and then regurgitated to feed comrades. Crop contents from a single forager in the genus *Formica* may be shared by an entire colony in a matter of hours (Wilson 1957), and Eisner and Happ (1962) suggest that similar distribution might be expected

among *Camponotus*. Repeated regurgitation and comrade feeding effectively filters out even small particles which are ultimately ejected as bucal pellets.

Fowler and Roberts (1980) distinguished ground foragers, tree foragers, and aphid guards. They found that 13 percent of their foragers had more than 1000 µg of carbohydrates per ant, as opposed to other foragers with 100 to 500 µg and aphid-guardians with 20 to 100 µg carbohydrate per ant. They suggest that foragers may stock up on carbohydrates as an energy source to sustain their wide-ranging activities while aphid guards need little reserve energy for their tasks. The foragers with high content of carbohydrates may be serving as walking tank-cars to transport carbohydrates from source, to the nest. Since foraging activity was most intense in late June and early July, it was probably correlated with brood maturation and the associated increased nutrient demands (Fowler and Roberts 1980).

Ayre (1963; 1967) suggested that enzymes present in the crop might contribute to digestion of solid food material either in the crop or when crop contents were regurgitated on the food material. Ayers found amylase present and used this as evidence that fungi are an important food source for carpenter ants. He observed *Camponotus* chewing on commercial mushroom slices and on cultures of three different fungi isolated from rotting wood. The ants regurgitated their crop contents on the chewed areas and reingested the liquid. Ayre (1963; 1967) felt this demonstrated that *Camponotus* use fungi as a food source.

Methods and Results

Carpenter ants *C. pennsylvanicus* and *C. pennsylvanicus ferruginia* are frequently seen on chestnut trees in Connecticut, with *C. pennsylvanicus ferruginia* being more common. These ants tend colonies of aphids on leaves *Colaphis castaneae* and on young stems *Petchia virginiana*. We have collected aphid guardians and foragers of both carpenter ant species in the past two years. We surface-sterilized some in 70 percent EtOH for a few minutes and removed their crops to 2 percent water agar. We washed others with sterile water and plated the wash-water on 2 percent water agar. The resulting fungal colonies were transferred to potato dextrose agar for identification. Among 24 aphid-guardians tested July through September, none had *Endothia parasitica* in their crops. Eight others had no *E. parasitica* on their surfaces. Among 19 foragers, four carried *E. parasitica* in their crops (June, 1980; June, 1981) and three carried *E. parasitica* on their bodies (July, 1981). Foraging and feeding observations were made on three sets of three trees with (a) *E. parasitica* H and V strains (two inoculations of each), (b) *E. parasitica* as in (a), but with molasses applied weekly to the tree trunk above the inoculation sites, and (c) control trees with sterile agar medium inoculated into four holes and with molasses as in (b). Many foragers *C. pennsylvanicus ferruginia* were observed in July and August 1981 chewing on cankers receiving the (a) and (b) treatments in approximately equal numbers (no counts were made). Ants were never observed chewing on the control trees.

Conclusions

Since carpenter ants were clearly carriers of *E. parasitica* because of both their feeding and nest building behavior, we will continue to capture them for analysis and observe them in our chestnut experimental plots. Because carpenter ants prefer aphid honey dew they minimize their feeding on other sources, such as fungi, when aphids are available. Therefore, we will concentrate next year on foragers in the early spring, before aphid colonies are established, and when nest building activity is highest.

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ASSOCIATION OF ENDOTHIA PARASITICA WITH MITES ISOLATED FROM CANKERS ON AMERICAN CHESTNUT TREES

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ABSTRACT.--*Cankers from American chestnut stump sprouts in Virginia plus two large, surviving American chestnut trees in Virginia and West Virginia were examined for the presence of mites (Acarina). All canker samples examined contained mites, with many in the families Oribatidae, Belbidae and Parasitidae. Further identification is in progress. One hundred and sixty-two mites from the stump sprouts growing in eight areas of Virginia were plated on acidified potato dextrose agar in an attempt to isolate Endothia parasitica from them. The blight fungus was recovered from 56 (34.6 percent) of the 162 mites and from at least one mite in all eight areas. These results suggest that mites may disseminate E. parasitica (including, possibly, hypovirulent strains) when they move from bark tissues into the soil and other trees.*

Introduction

It is possible that a carrier may be important in the dissemination of hypovirulent strains of *Endothia parasitica* in North America. This study attempted to provide insight into the possibility that mites may act as carriers of *E. parasitica*. Our objectives were to determine if mites (members of the order Acarina) could be found in association with cankers of American chestnut (*Castanea dentata*) and if *E. parasitica* could be isolated from any mites found.

Methods

Endothia parasitica cankers from five American chestnut stump sprouts in the Johns Creek area of the Jefferson National Forest, Virginia, plus two large, surviving American chestnut trees in Virginia and West Virginia were excised and examined to determine if mites occur commonly on cankers. Also, decayed bark material, which was obtained from the bases or crotches of American chestnut trees, was examined. Cankers (often an attempt was made to excise the entire canker) were obtained from trees with a hammer and a

chisel. All cankers from a given area were collected in a single plastic bag. Canker materials were stored under refrigeration until examined under the dissecting scope. Decayed bark material from the base or crotches of the two large, surviving American chestnut trees and five stump sprouts was placed in a Berlese funnel, equipped with 40-watt incandescent light bulbs, for 72 hours. Mites were collected in a jar of ethanol at the base of the funnel.

All canker surfaces were found to contain mites as did all decayed bark material samples. Five to 10 mites from each canker or bark material sample were mounted as slides for identification. Initially, attempts were made to identify, at least to family, the more commonly found mites.

Canker materials were excised from 5 to 15 diseased American chestnut stump sprouts from each of 8 areas in Virginia to determine if mites could carry *E. parasitica* propagules in or on their bodies. All mites found on these diseased tissues were transferred, aseptically, to acidified, potato-dextrose agar (APDA). An effort was made to keep the mites alive during transfer. A total of 162 mites were plated. Fifty-six or 34.6 percent produced *E. parasitica* on APDA. One to 12 mites at each location were associated with *E. parasitica*. Seven of the most commonly encountered, but apparently different mites, were identified to family. The three families were Belbidae, Oribatidae and Parasitidae, suggesting that a search for a suitable carrier might begin with members of these families.

Discussion

There are several characteristics regarding mites which would be beneficial should they be found to be carriers. Many species commonly found in association with forest litter and plant material in general are fungus feeders: this is especially true of the orbatids. If a monophagus species which feeds on *E. parasitica* could be found, it would be especially beneficial in a carrier capacity, from the standpoint of its need to search for suitable food sources such as *E. parasitica* and presumably cankers on American chestnut. Thus, mites may be important in local spread of *E. parasitica* from canker to canker on a single tree or among cankers on neighboring trees.

Another characteristic of mites which may be particularly beneficial in a carrier relationship is the intimate contact in which mites live in association with the bark surface. In the course of this study, mites were most often found in moist fissures and natural cracks in the bark surface, placing them in areas where infection is most likely to occur. The small size of these Arthropods allows them easy access to suitable infection courts. It should be noted that many insect species (orders Coleoptera, Lepidoptera) which have been associated with chestnut blight cankers go through several life stages or what is known as complex metamorphosis. One of the evolutionary advantages of such species is that not all developmental stages are found in the same habitat or require the same food source. This is good for survival purposes in that different stages of the same species do not compete for the same food, but a disadvantage in terms of carrier efficiency in that the potential carrier is only in contact with the desired host for part of its life. Mites undergo simple metamorphosis and in most cases nymphal stages and adults share the same habitat and food source, placing them in contact with the desired host for much of their life cycle.

A third and perhaps more important aspect regarding mites as potential carriers is that they may produce several generations per year, unlike many insect species which have been associated with cankers caused by *E. parasitica* on American chestnut. Mites continue to multiply throughout the growing season.

Probably the most limiting factor regarding mites as potential carriers is their mobility. This may be a serious restriction to widespread rapid dissemination of hypovirulent strains, although their movement may exceed the rate of spread of hypovirulent strains of approximately 1 meter per year which Grente and Berthelay-Sauret (1978) reported in Europe. Mites may also be windblown or carried on animals, birds, or beetles which come in contact with them, facilitating greater movement.

We do not know the type of propagule that was being carried by the mites we plated. This may be an important consideration for we know that the most debilitated hypovirulent strains of *E. parasitica* sporulate poorly. At this point, we cannot say if mites carry hypovirulent *E. parasitica* for we did not carry out pathogenicity tests on any of the strains recovered from the mites we plated. However, the possibility that mites are carriers of hypovirulent strains appears to warrant further investigation based on these data which have indicated that mites are capable of carrying *E. parasitica* in some form.

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NATURAL DISSEMINATION OF ARTIFICIALLY INOCULATED HYPOVIRULENT STRAINS OF ENDOTHIA PARASITICA

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ABSTRACT.--*This study was designed to determine if hypovirulent (H) isolates of Endothia parasitica could be recovered from cankers that developed naturally on American chestnuts Castanea dentata previously inoculated with H strains. Isolates from 19 percent (89) of 475 cankers that developed on 220 trees over a 2½-year period displayed morphological characteristics that were different from wild-type virulent (V) strains. These atypical isolates were separated into six morphological groups. A representative isolate from each group was able to alter the morphology of several V strains when grown together in vitro. Double-stranded RNA was detected in the representative isolates from four of the six groups. The results of these tests and their morphological similarity to known H strains indicate that isolates in at least four of the six groups are H. When cankers that yielded H isolates were extensively sampled, several different H types and V isolates were sometimes recovered from a single infection.*

Introduction

Hypovirulent (H) strains of the chestnut blight pathogen, *Endothia parasitica*, are believed to be responsible for the natural control of chestnut blight that has occurred on European chestnuts, *Castanea sativa*, throughout Italy (Grente and Berthelay-Sauret 1978; Turchetti 1978) and on American chestnuts, *Castanea dentata*, in several areas of Michigan (Weidlich this proceedings). They have been used to biologically control cankers incited by wild-type virulent (V) strains (Van Alfen et al. 1975; Grente and Berthelay-Sauret 1978). Establishment of a self-perpetuating H based control process within the native range of the American chestnut would require that H strains disseminate throughout the existing V population of *E. parasitica* (Day 1978). Large abnormal cankers, found in areas where remission of chestnut blight has been observed, could be important to dissemination by providing reservoirs of persistent H inoculum (Elliston 1982). This paper reports on attempts to artificially establish large H cankers on American chestnuts and determine if H strains were present in naturally occurring cankers that developed on the same trees.

Materials and Methods

In May, 1979, two 0.1 hectare study plots with abundant American chestnut regeneration were established in 10- to 15-year-old cut-over areas near

Parsons and Bartow, West Virginia. Chestnut stems ranged in size from 3 to 11 cm, 1.3 m above the ground. The plots were cleared of competing vegetation and trees with visible *E. parasitica* cankers removed leaving uninfected individuals for the study.

Inoculum preparation and inoculation procedure. Eleven hypovirulent (H) strains of *E. parasitica* representing collections from France, Italy and the United States were used as inoculum (Table 1). Each strain was grown at 25 C in two 1000 ml Erlenmeyer flasks that contained 300 ml semi-solid glucose yeast extract (GYE) agar medium. The GYE medium contained 10.0 g glucose, 2.0 g Difco Bacto yeast extract, 1.0 g of KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.2 mg of Zn^{++}, 0.1 mg of Mn^{++}, 0.1 mg of thiamine hydrochloride, 0.01 mg of biotin, 4.0 g of Difco Bacto agar, and 1000 ml of distilled H_2O (Lilly and Barnett 1951). After 10 days growth, the contents of both flasks were combined and mixed in a waring blender to make an agar slurry.

Table 1. Hypovirulent strains of *Endothia parasitica* inoculated into wounded American chestnuts

Strain origin	Strain designation
France	JR 4[a/], 43[b/], A-1[b/]
Italy	27-10[a/], 54-2[a/], 50[a/], Euro 11[c/], Euro 7[c/]
United States	60[a/], 209[a/], 206[a/]

a/ Obtained from the Connecticut Agricultural Experiment Station, New Haven, CT 06504

b/ Isolated from virulent cankers on American chestnuts inoculated with hypovirulent strains; *in vitro* characteristics resembling French hypovirulent strains (Grente and Berthelay-Sauret, 1978)

c/ Isolated from abnormal cankers on European chestnuts in Italy

Twenty-five cm wounds encircling half the circumference of the study trees were made by "scratching" the bark with metal wood screws mounted in a wooden board. Wounds were made at ground level, 1.5 m and 3.0 m above the ground on each tree. The same individual hypovirulent strain was introduced into all three wounded areas on a tree by spreading the agar slurry over the wounds with a 6 to 8 cm wide brush. The wounds were covered with clear plastic (0.5 mil) and sealed with masking tape for approximately 30 days. All strains were inoculated into 10 replicate trees in each study plot so that a total of 220 trees were used.

Isolations. When isolations of mycelium were made from cankers, 0.5 cm diameter bark plugs were removed with a leather punch. Plugs were surface sterilized in a 0.5 percent sodium hypochlorite solution for 10 minutes,

washed in distilled H_2O and placed in petri plates containing GYE medium amended with 2 percent agar, streptomycin (6 mg/l) and chlorotetracycline (50 mg/l). Mycelial transfers were made from the advancing edge of cultures from each plug and placed in petri plates containing Difco potato dextrose agar medium (PDA) amended with biotin (5 µg/l) and methionine (100 mg/l). Two transfers were placed at maximum spacing on each plate, incubated at 25 C in a 16-hr photoperiod (white fluorescent light) for 7 to 10 days and then morphologically evaluated.

Canker survey and isolate designations. Trees in both study plots were surveyed every 60 days between April and December for the presence of *E. parasitica* cankers in nonwounded areas. When cankers were initially observed, isolates of *E. parasitica* were obtained from five different points and classified as morphologically resembling wild-type V strains (Elliston 1978) or showing atypical characteristics. Atypical isolates were considered to be potentially H and grouped according to their morphological similarity.

Extensive sampling. Several cankers in both study plots, from which suspect H isolates had been obtained, were selected for extensive sampling. This included cankers that yielded isolates from each suspect H morphology group and only those not associated with wounded areas or other cankers. Bark plug samples (20 to 24) were removed at 2 to 3 cm intervals near the canker margin and toward the canker center if bark tissue was present. Each plug and the resulting isolates were numbered according to their position in a canker so that the distribution of morphology types could be determined.

Conversion tests. One isolate from each suspect H morphology group was tested for its ability to alter the morphology (convert) of V strains when paired *in vitro* (Anagnostakis and Day 1979). Each type was paired against V strains from 34 different vegetative compatibility (v-c) groups (MacDonald unpublished data) on amended Difco PDA medium at 25 C in a 16-hr photoperiod for 7 to 10 days. Developing V colonies were observed for evidence of conversion. Mycelial transfers were made from V strains that displayed morphological changes to amended PDA medium and grown at 25 C in a 16-hr photoperiod to confirm conversion.

DsRNA analysis. Suspect hypovirulent isolates that were used in the conversion tests and a known V strain were screened for the presence of double-stranded ribonucleic acid (dsRNA). The V strain was included as a control for the extraction process because dsRNA has not been reported in V strains (Day et al. 1977). Mycelia for extraction were grown in six 1000 ml Erlenmeyer flasks containing 125 ml GYE liquid medium at 25 C for 10 days. The mycelial contents from all six flasks of a single isolate were combined and press-dried between layers of absorbent towels. Mycelial pad weights ranged between 3.2 to 5.5 g. Nucleic acids were extracted by methods previously described (Morris and Dodds 1979) with the exception that mycelium was frozen in liquid nitrogen and pulverized with a cold mortar and pestal. The dsRNA was purified according to the procedure described in method 1 of Morris and Dodds (1979) with the addition of a second CF-11 chromatography step. Extracts containing dsRNA were dissolved in 50 µl electrophoresis buffer (0.09 m Tris, 0.09 M Boric Acid, 0.001 EDTA, pH 8.3) containing 10 percent sucrose, electrophoresed on 5 percent polyacrylamide gels for 10 hr at 10 v/cm and stained with ethidium bromide (5 µg/ml). Ribonuclease digestions were conducted to verify the presence of dsRNA (Morris and Dodds 1979).

Results

During the two-to-three months following inoculation, the host formed callus in the wounded areas and in adjacent portions of the stem that were covered with plastic wrap. In some cases this caused the entire wrapped area to appear swollen. This did not occur in other areas where the bark was unwounded.

Growth of *E. parasitica* from the inoculation sites was indicated by the expansion of necrotic areas in the bark. This was primarily observed at wound sites inoculated with the Italian strains (Table 1) and American strains 209 and 206. However, sustained fungal growth was not usually observed. Limited numbers of pycnidial stromata were formed in these same areas during the first growing season, but usually within a relatively small portion of an inoculation site.

Canker survey and isolate designations. Between May, 1979 and November, 1981, 475 naturally occurring cankers developed in the uninoculated bark tissue of study trees at both locations. Canker incidence was greatest (70) for trees inoculated with strain A-1 and least (19) on trees that received 206 inoculum. Trees inoculated with other H strains had comparable numbers of infections (Figure 1).

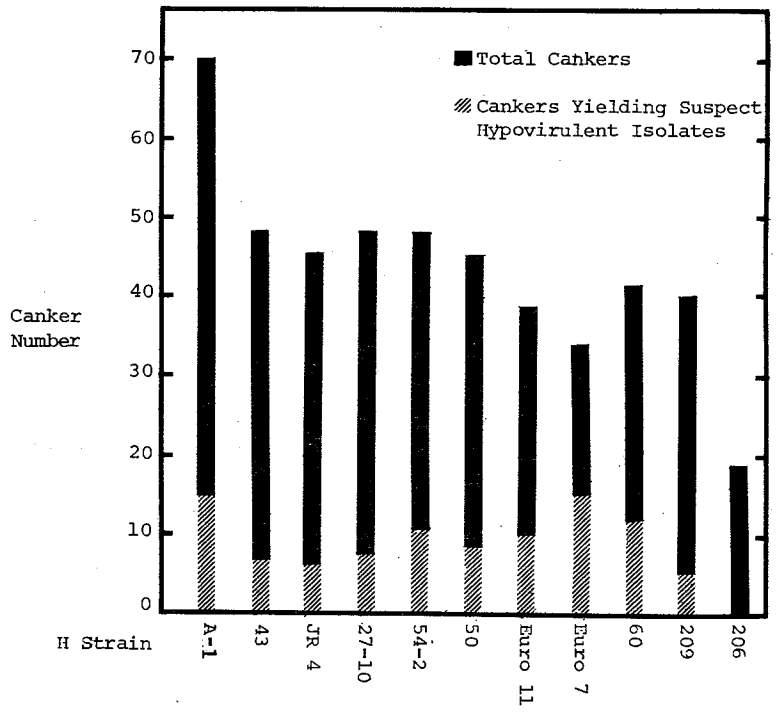


Figure 1. Incidence of naturally occurring cankers and number yielding suspect hypovirulent isolates on American chestnuts inoculated with hypovirulent strains of *Endothia parasitica*.

Eighty-one percent (386) of the naturally occurring cankers yielded only colonies that resembled wild-type V strains. Isolates from the remaining 19 percent (89) displayed atypical morphologies which were considered to represent suspect H types. Suspect H isolates were recovered from cankers on several replicate trees for each inoculum type except 206 (Figure 1). All suspect H isolates from a tree did not always morphologically resemble the H strain that had been inoculated on the tree.

Six distinct suspect H morphology groups were established based on the similarity of isolates in culture (Table 2). Isolates classified in group 1 were recovered most frequently (54 percent) from cankers that yielded suspect H isolates. Group 1 and 2 isolates resembled inoculated H strains 27-10, 54-2, 50, Euro 11, and Euro 7 while group 3 resembled strains 43 and A-1. Group 5 was similar to strain JR 4 except for the presence of curved aggregates of hyphae within the colony. Groups 4 and 6 were not morphologically similar to any of the inoculated H strains.

Table 2. Morphology and frequency of suspect hypovirulent *Endothia parasitica* isolates obtained from naturally occurring cankers

Suspect hypovirulent group	Cultural morphology	Frequency	
1	radially symmetrical growth; white mycelium; yellow-pigmented colony center	48[a/]	(54)[b/]
2	radially symmetrical growth; white	5	(5)
3	slow, erratic growth; white mycelium	5	(5)
4	slow, erratic growth; orange-pigmented mycelium	2	(2)
5	radially symmetrical growth; orange-pigmented mycelium; aggregates of curved hyphae within the colony	8	(9)
6	erratic growth; orange-pigmented mycelium	21	(24)

[a/] Total cankers that yielded isolates resembling the suspect hypovirulent morphology group

[b/] Percent of all cankers (89) that yielded suspect hypovirulent isolates

Extensive sampling. Twenty-three cankers, from which isolates in groups 1, 2, 3, 5 and 6 had been obtained, were extensively sampled within 20 to 24 months after they were first detected (Table 3). Group 4 cankers were not

Table 3. Morphology groups of *Endothia parasitica* isolates obtained from extensively sampled cankers on American chestnut

Canker	Initial isolate morphology groups	Extensive sample groups	Canker	Initial isolate morphology groups	Extensive sample groups
6-3P[a]	5[b]	6[b] (8)[d] wt[c] (4)	56-1P	3	1 (13) 2 (2)
8-3P	6	6 (11) wt (4)	69-1P	6	6 (7) wt (9)
10-1P	2	2 (10) 3 (1) wt (5)	72-1P	2	6 (3) wt (4)
			89-1P	1	1 (3)
16-2P	1	6 (12) wt (2)			3 (10) wt (1)
26-1P	1	1 (6) 2 (2) 3 (4) wt (1)	100-3P	1	1 (12) 2 (1) 3 (5) wt (1)
34-1P	1	1 (5) 3 (1) 6 (2) wt (8)	1-1B	1	1 (16) wt (1)
			10-3B	1	1 (7) wt (11)
37-3P	1	1 (10) 2 (2) 3 (8) 4 (1) wt (1)	17-1B	1	wt (22)
			49-2B	5	1 (2) 5 (15) wt (2)
37-4P	1	1 (15) wt (2)	75-1B	1	1 (17) wt (1)
42-1P	1	1 (22)	87-2B	1	1 (12)
42-2P	1	1 (16) 4 (2) wt (1)			3 (9)
50-1P	1	1 (14) wt (2)	95-2B	1	1 (15) wt (1)

[a] Letters indicate Parsons or Bartow study plot locations.
[b] Number corresponds to suspect hypovirulent morphology group listed in Table 2.
[c] Morphology designation for isolates resembling wild-type virulent strains of *E. parasitica*.
[d] Number in parenthesis indicates total number of samples that yielded each morphology group.

sampled because they had coalesced with other infections by the time extensive sampling was conducted. Group 1 cankers were predominately selected because more were available that had not coalesced with other infections.

Results of the sampling showed that isolates resembling several suspect H morphology groups and wild-type V strains could be present in the same canker (Table 3). From most group 1 cankers, isolates with group 1 morphology were recovered except for cankers 89-1P and 10-3B where other morphology groups predominated. Group 2, 3, 5 and 6 cankers gave fewer isolates that resembled the initial type although isolates from group 5 canker 49-2B predominately resembled the initial group 5 isolate (Table 3). Reconstruction of the cankers based on isolate morphology showed that specific morphology types usually occurred in several areas of a canker as illustrated by canker 37-3P (Figure 2).

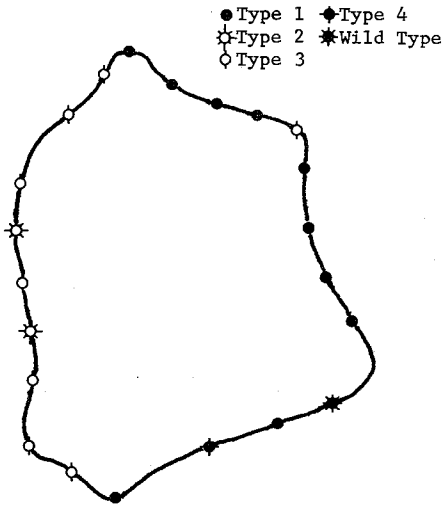


Figure 2. Distribution of *Endothia parasitica* morphology types in naturally occurring canker 37-3P.

Conversion tests. A representative isolate from each suspect H morphology group (Table 2) was able to convert V strains from 3 to 5 different v-c groups (Table 4). In all cases, the converted V strain morphologically resembled the suspect H isolate with which it was paired.

DsRNA analysis. The six suspect H isolates used in the conversion tests were tested for the presence of dsRNA. The dsRNA patterns observed from these isolates and a V strain after ribonuclease treatment in 0.3 M NaCl are shown in Figure 3. Banding patterns before and after ribonuclease treatment were the same for gels 1, 2, 3 and 4. Gels 5 and 6 each contained a single band following electrophoresis that disappeared after ribonuclease treatment in 0.3 M NaCl. All fluorescence disappeared from gels 1, 2, 3 and 4 after incubation with ribonuclease in water verifying the presence of dsRNA.

The isolates from suspect H groups 1, 2, and 3 (Table 2) contained both major and minor segments of dsRNA while the type 4 isolate contained a single major segment (Figure 3). Molecular weights of the segments were not estimated.

Table 4. Virulent strain campatibility groups of *Endothia parasitica* that were converted by suspect hypovirulent isolates in six morphology groups

Isolate	Suspect hypovirulent morphology group	Virulent compatibility groups converted
37-2P	1	A[a/], Y, Z, Delta
99-2P	2	E, Delta, Gamma
5-1P	3	A, E, Y, Z, Gamma
91-1P	4	F, J, Q, Alpha, Iota
64-2P	5	L, Delta, Gamma
10-4P	6	Y, Epsilon, Gamma

[a/] Indicates different compatibility groups (MacDonald unpublished data)

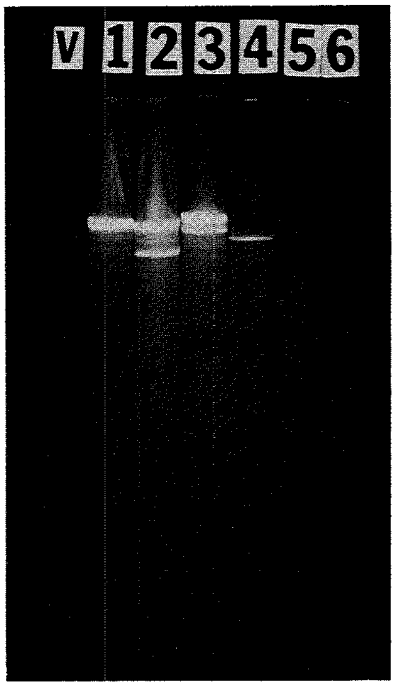


Figure 3. Polyacrylamide electrophoresis gels showing dsRNA banding patterns from suspect hypovirulent morphology groups 1-6; Gel on the extreme left is from a wild-type virulent strain.

Discussion

The majority of the suspect H isolates displayed morphological characteristics similar to those previously described for H strains (Van Alfen et al. 1975; Grente and Berthelay-Sauret 1978; Elliston 1978; 1982). The ability of isolates from each suspect H group to convert the morphology of several V strains is consistent with the *in vitro* behavior of known H strains (Anagnostakis and Day 1979). However, the failure to detect dsRNA in group 5 and 6 is inconsistent with the findings from other H strains (Day et al. 1977). Previous dsRNA extractions have indicated that dsRNA recovery from American H strains is variable (Willey 1980; Elliston personal communication). This could be related to a lower amount of dsRNA (Dodds 1980) and may explain the inability to recover dsRNA from the group 5 and 6 isolates. Extracting from larger amounts of mycelium might enhance the recovery of dsRNA (Elliston personal communication).

At present, results from the morphology evaluations, conversion tests and dsRNA analyses indicate that suspect H isolates in groups 1, 2, 3 and 4 are H. The morphological similarity of groups 1, 2 and 3 to several of the inoculated H strains and their isolation from subsequent naturally occurring cankers suggests that isolates in these groups originated from the H strain inoculation sites. The lack of resemblance between group 4 isolates and any of the inoculated H strains is yet unexplained. However, their origin must also be related to the H strain inoculation sites because morphologically atypical isolates have never been recovered during previous isolations from several hundred cankers in areas of West Virginia where H strains have not been introduced (MacDonald unpublished data).

The occurrence of H isolates in naturally developing cankers is encouraging because it shows for the first time that artificially introduced H strains can disseminate on American chestnuts. The lack of natural dissemination by H strains in previous field control studies (Jaynes and Elliston 1978; MacDonald and Double 1981) may have been due to the use of individual or mixtures of H strains that exhibited low pathogenic and sporulation capabilities (Elliston 1978; Jaynes and Elliston 1980). However, these studies did not report any attempts to obtain *E. parasitica* isolates from cankers that developed following the introduction of H strains. The presence of H isolates in the present study could only be determined by obtaining *E. parasitica* isolates and morphologically evaluating them. This suggests that dissemination of H strains and recognition of hypovirulence in cankers may initially only be verified by sampling.

The incidence of naturally occurring H isolates in this study must be regarded as a minimum number that exists in the study areas. The initial screening of cankers by sampling at only five points may not have been sufficient to detect H isolates in all cankers. Cankers that failed to yield atypical isolates were considered to be V. Yet, the extensive sampling of cankers that contained H isolates showed that both H and V isolates could be present in a single canker. Resampling the V cankers might lead to the recovery of additional H isolates.

The inoculation procedure did not establish abnormal cankers like those found in Italy or Michigan. Canker development of this type may only result from a long-term interaction of H strains with V strains and the host

(Elliston 1982). However, the procedure was successful in establishing H strains and may have helped to promote their natural dissemination. The presence of a disseminating H population has not yet led to noticeable changes in disease incidence on the study trees as V strains continue to cause significant mortality. If the transition of V to H in the *E. parasitica* population is to occur, it may require more than one generation of chestnut sprouts before H strains predominate.

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ATTEMPTS TO CONTROL CHESTNUT BLIGHT WITH SLURRY AND CONIDIAL SPRAYS OF HYPOVIRULENT STRAINS

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ABSTRACT.--*Four field plots were established in 1978 in forested areas with high densities of native chestnut sprouts. Treatments were repeated each year for four years and consisted of mist-blowing solutions of conidia of cytoplasmic hypovirulent (CH) strains and inoculating natural cankers with slurries of CH strains. Treated areas consisted of all the sprouts within a 25 m radius. Competing hardwoods were cut or killed. Stems in the 25 to 50 m radius have been monitored for control of the blight and spread of CH strains from the treated center. Data on stem survival, stem size, canker persistence, new cankers, and canker location are being obtained. Preliminary results will be presented. Inoculation with CH slurries is keeping stems alive longer. There is some evidence of the establishment of persistent cankers within the treated area as well as in the outer perimeter. However, new infections of normal strains are prevalent in both the inner treated area and the outer untreated area.*

Control of virulent (normal, V) infections of *Endothia parasitica* on American chestnut by inoculation with agar slurries and even conidial sprays of hypovirulent (H) strains has been previously demonstrated (Jaynes and Elliston 1978; 1980). However, natural spread of the control agent(s), giving protection from secondary infections on the same or neighboring stems, has not been clearly shown. This failure of hypovirulent spread in the eastern United States, in contrast with the situation in Italy and France, has been attributed to: 1) a difference in the host species, *Castanea dentata* vs. *C. Sativa*; 2) a smaller density and stem diameter of host trees; 3) a lack of appropriate vectoring mechanisms; 4) use of the wrong combination of hypovirulent strains; 5) other differences in the parasite, including more vegetative compatibility groups and an increased ability to produce perithecia in the United States (Elliston 1981; 1982); 6) an overabundance of natural V inoculum compared to the small quantities of H inoculum introduced into test plots to date. Some of these problems might be overcome by establishing long-term test plots in areas with high densities of American chestnut, where mixtures of hypovirulent strains could be introduced repeatedly and any competing hardwoods could be removed. Any spread of H strains could be monitored from the central treatment area.

Materials and Methods

In 1978 four field plots were established in northeastern Connecticut in forested areas with high densities of native chestnut sprouts. Partial cutting of the overstory hardwoods had previously occurred in all plots. Hypovirulent treatments were repeated each year for 4 years and consisted of mist-blowing solutions of conidia into the plot and/or hand inoculating natural cankers with slurries of H strains. All treatments were during the growing season (May to October). The first year the stems in all four plots were examined three times (June, July, and September) and all cankers detected within 2¼ m of the ground were inoculated with a slurry of H strains. The combinations of sprays and slurry inoculations were different for each plot (Table 1). One plot, TF, received no spray treatments.

Table 1. Hypovirulent treatments of four plots for 4 years.

Plot	Treatments[a/]	Year and number of treatments				
		1978	1979	1980	1981	Total
GS	Slurry 5	3	1	1	1	6
	Spray 5	1				1
GN	Slurry 10	3		1	1	5
	Spray 5	1	2	1	1	5
TF	Slurry 5	3	1	1	1	6
TN	Slurry 10	3		1	1	5
	Spray 10	2	2	1	1	6

[a/] Cultures of 5 stocks included: EP-4 French, 43 French-American, 47 Italian, 60 American, 172 American. Cultures of 10 stocks included, in addition: EP-49 Italian, 51 Italian, 88 American, 92 American, 171 American.

Prior to the first treatment, five normal cankers per plot were sampled and typed for vegetative compatibility (v-c). The 20 cankers represented 16 v-c Types (4, 8, 9*, 10*, 11, 18, 19*, 20, 24*, 26, 30, 41, 43, 44, 67, 70-those with an asterisk were isolated from two cankers). The H strains were of French, Italian, and American origin and included a wide range of pathogenicities. Seven of the 10 H strains have been typed and six v-c groups are represented (5, 9, 10, 11, 12, 13-two in group 12).

Treated areas consisted of an inner circle of 25 m radius (0.2 ha). At the start of the experiment, competing hardwoods were killed and American chestnut stems girdled with chestnut blight were cut and removed. All live American chestnut stems 2.5 cm in diameter or larger at 135 cm above ground within the 25 m radius were included in the treated area. American chestnut stems in the 25 to 50 m radius (0.6 ha) were also monitored for control of the blight and spread of H strains from the treated center. Data on stem survival, stem size, canker persistence, new cankers, and canker location were obtained in each of the 4 years from the inner treated and outer untreated circles.

The culturing and inoculating techniques of the H strains were as previously reported (Jaynes and Elliston 1978; 1980). Slurries were inoculated around the periphery of each canker. Conidia were sprayed from a back-pack mist-blower, 9 liters/plot. The total number of conidia sprayed in each treatment ranged from 1.2 to 12.1 x 10^{11}. With few exceptions concentrations of conidia of the component strains were within 4 to 5 fold of each other, e.g. 3 to 15 x 10^{7} per ml.

Endothia parasitica was isolated from a few persistent and apparently healing cankers present in the outer circle. These isolates were characterized for morphology on potato dextrose agar and two were tested for dsRNA as previously described (Dodds 1980; Scharf and DePalma 1981).

Results

There are many parameters that need to be analyzed including survival of chestnut stems, changes in basal area, number and location of cankers, persistence of cankers, persistence of infected stems, and isolation and characterization of H strains beyond the treated area. The following results are preliminary and incomplete.

The percentage of stems surviving at the end of 4 years is presented in Figure 1. As expected, a significantly higher percentage of stems have survived in the inner treated circle compared to the outer untreated circle. Average survival in the inner circle was 81 percent compared to 58 percent for the outer. Average stem diameter over 4 years has increased more for stems in the inner circle, 46 percent compared to those in the outer, 12 percent (Figure 2).

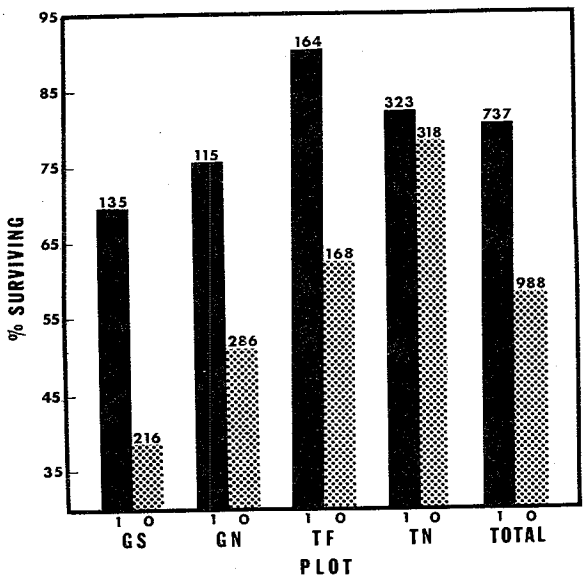


Figure 1. Percentage of stems surviving in 1981 for the inner treated (I) and outer untreated (O) areas of each plot based on the total number of stems that attained at least 2.5 cm d.b.h. in the 4-year period. Total stems indicated at the top of the bars.

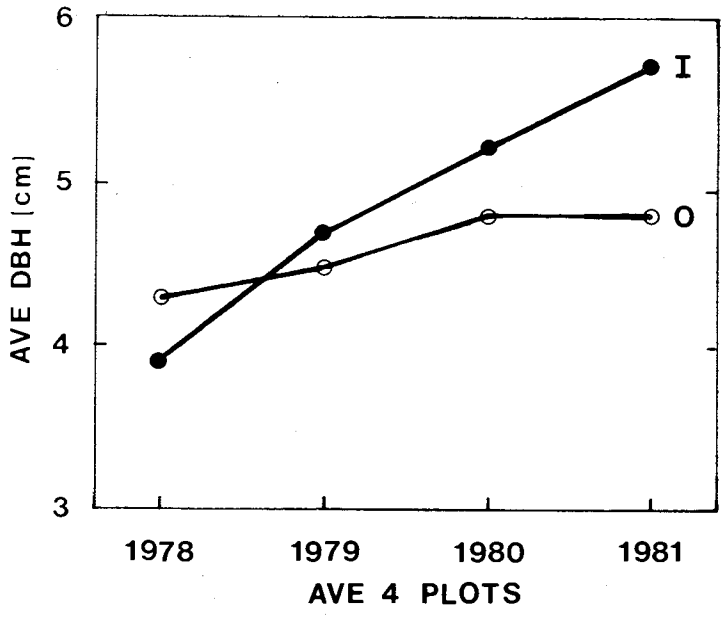


Figure 2. Average stem diameter of American chestnuts in the four inner (I) treated and four outer (O) untreated plots.

The percentage of stems surviving in 1981 in each successive 5 m annulus of the inner and outer circles is shown in Figure 3 for 1 and 3 years. The lines represent the best fit for inner and outer circles. Except for plot GN survival in the inner treated circles was unrelated to distance from the plot center. Survival in the untreated outer circle generally decreased with increasing distance from the treated area. However, survival in the innermost annulus (26 to 30 m) of the untreated outer circle tended to be the same as survival within the treated circle.

Isolations taken from two persistent cankers outside the 25 m radius of plot TN (at 33 m) were tested for dsRNA. One isolate contained dsRNA, the other did not. This tree has been girdled with a superficial canker since 1980 but was still growing vigorously in 1981. Isolates have been obtained from other "healing" cankers in the untreated outer areas but have not been tested for pathogenicity or dsRNA.

Discussion and Conclusions

Inoculation of natural cankers on American chestnut with mixtures of H strains significantly prolongs the survival of the chestnut. The H strains are having an apparent positive effect beyond the treated area.

The tendency for higher survival in the portion of the untreated outer circle nearest the treated circle suggests that hypovirulent strains are moving slowly outward from the treated inner circle. The isolation of one strain containing dsRNA from the outer circle supports this suggestion, although it can not be unequivocally stated that no dsRNA strains were present prior to treatment in 1978. The better survival could also result, not from spread

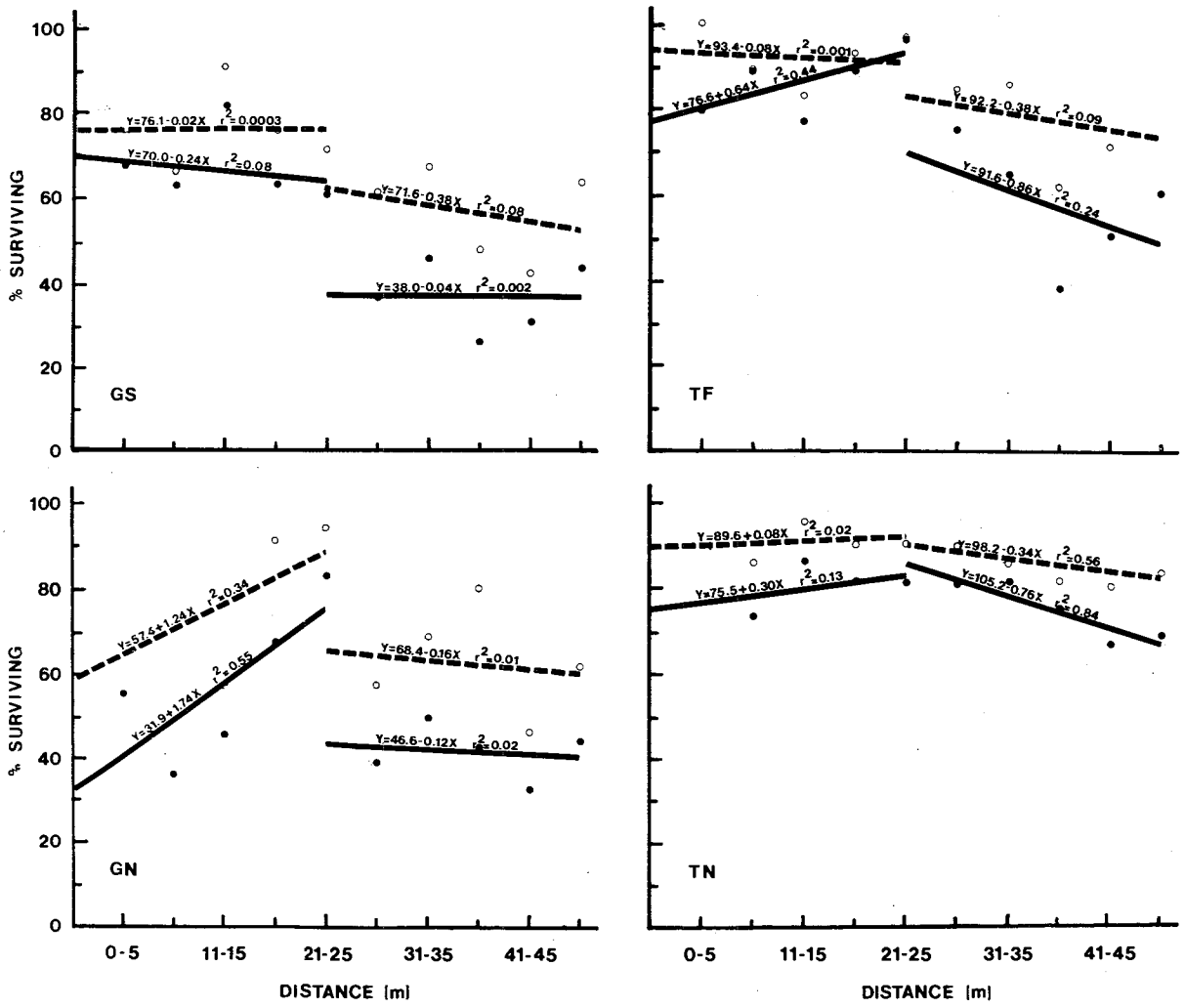


Figure 3. Percentage of stems surviving in 1981 in each section of 5 m radius from 0 to 50 m for each of the four plots based on the total number of stems that attained at least 2.5 cm d.b.h. Three-year (1978 to 1981) data indicated by closed circles and solid lines and 1-year (1980 to 1981) data by open circles and dashed lines.

of H strains, but from reduced virulent inoculum from the treated inner circle. Competition from hardwoods may also be a significant factor in the outer areas of the plots.

On plot GN the generally increased survival with increasing distance from center of the treated circle may simply be due to the small number of stems in the center of this plot. (The only stems lost to vandalism were in the center of plot GN.) Because the treatments for each plot differed (Table 1), it is not possible to determine whether a particular slurry or conidial spray was more effective.

This experiment was designed to determine natural spread from a central treated area. The results have not been dramatic but suggest that spread may be occurring. Even in France, spread of H and control of untreated

cankers is only first observed about 5 years after treatment (Grente and Berthelay-Sauret 1978). No further H treatments are planned for at least three of these four plots, but we expect to continue to observe the trees for survival and monitor spread of H strains.

Better combinations of H strains and application techniques are required for practical control of cankers on treated stems and subsequent spread of the controlling agents. The control observed in these plots can be attributed to the high density of stems, elimination of competition, and repeated inoculation with a mixture of strains.

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EFFECTS OF SELECTED NORTH AMERICAN AND ITALIAN CYTOPLASMIC HYPOVIRULENCE AGENTS ON NORTH AMERICAN AND ITALIAN STRAINS OF ENDOTHIA PARASITICA

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ABSTRACT.--*Each of four North American and two Italian cytoplasmic hypovirulence (CH) agents were transmitted on agar into each of 22 isolates of Endothia parasitica. Half of the isolates were from North America and half from Italy. Eleven vegetative compatibility groups were represented. Three of the isolates from North America and five from Italy had previously been naturally infected with CH agents and freed of these by single conidial isolation. The others had no known history of natural infection with CH agents. Each agent caused a characteristic disease in each isolate, regardless of geographic origin or previous history.*

Introduction

Cytoplasmic hypovirulent (CH) strains of the chestnut blight fungus, *Endothia parasitica*, studied to date contain double-stranded RNA (dsRNA), have abnormal cultural characteristics, and reduced abilities to cause chestnut blight (Day et al. 1977; Elliston 1978; Dodds 1980). Because dsRNA is characteristic of fungal viruses (Lemke and Nash 1974), virus-like agents are assumed to be involved.

The abnormalities found among CH strains are diverse. Previous work suggests that a variety of virus-like CH agents may contribute to this diversity (Dodds 1980; Elliston unpublished data). Differences in responses of strains to individual CH agents may also contribute to the diversity. This study was conducted to explore this latter aspect of the diversity question. In this work, the effects were determined by introducing four American and two Italian CH agents individually into representative American and Italian strains.

Materials and Methods

Cytoplasmic hypovirulence agents. Table 1 lists the CH agents used, their sources, and the effects they had on cultural characterisitcs and pathogenicity of the source strains. All but H_{I1} behaved as single agents; H_{I1} appears to be a mixture that includes H_{I2}.

Table 1. Sources and effects of six CH agents on cultural characteristics and pathogenicity of source strains.

CH agent	Source strain	Geographical origin	Cultural characteristics: Radial symmetry[a]	Radial striations[b]	Concentric rings[c]	Growth rate[d]	Pathogenicity[e]
H_{M_1}	EP-60	Michigan	-	-	-	+	-
H_{M_2}	EP-60	Michigan	+	+	+	++	++
H_{T_2}	EP-234	Tennessee	-	-	-	+	+
H_{V_1}	EP-700	Virginia	-	-	-	+	-
H_{I_1}	EP-419	Italy	+	-	±	++	++
H_{I_2}	EP-419	Italy	+	+	+	++++	+++
None			+	+	+	+++	+++

[a] + = radially symmetric; - = radially asymmetric.

[b] + = radially striated; - = not radially striated.

[c] + = concentric rings present; - = concentric rings absent.

[d] + = slow; ++ = moderate; +++ = fast; ++++ = very fast.

[e] - = nonpathogenic; + = very weakly pathogenic; ++ = moderately pathogenic; +++ = highly pathogenic.

Strains of *Endothia parasitica* and cultural conditions. Table 2 lists the 22 strains of *E. parasitica* used as recipients of CH agents, their geographical sources, vegetative compatibility (v-c) group, and other pertinent information. Eight of the strains were normal single conidial isolates of strains naturally infected with CH agents.

Stock cultures were maintained on slants of Difco potato dextrose agar amended with 0.1 g L-methionine and 0.1 mg biotin per liter (PDAmb) at 4 C. Colonies used for inoculum were grown on 20 ml PDAmb in 100 x 15 mm petri dishes for 7 to 9 days at 20 C under fluorescent lights with a 16 hr photoperiod (standard conditions). Plates were sealed with a single layer of parafilm and inverted under the lights.

Determination of cultural characteristics. Cultural characteristics were determined with 7-day-old colonies grown under the conditions described. Inoculum for these colonies consisted of 8 mm diameter plugs cut with a sterile cork borer from the advancing margins of inoculum colonies. Four cultures of each strain were grown and examined.

Transmission experiments. The CH agents were transmitted from donor strains (those containing CH agents) to recipient strains (those lacking CH agents) on PDAmb. Donor and recipient strains in the same v-c group were paired on 100 x 15 mm plates containing 20 ml agar. Those in different groups were paired on 150 x 15 mm plates containing 40 ml agar to permit more extensive interactions. A vertical line was drawn with a marking pen across the bottom of each plate through the center. In most experiments, three donor inoculum blocks were placed near the center ca. 5 mm apart and 2 mm to the left of the line, with mycelium facing down and leading mycelium directed toward the line. Recipient inoculum blocks were placed similarly opposite the donor blocks ca. 2 mm to the right of the line. Colonies of donor and recipient strains used as controls were grown with only donor or recipient inoculum arranged as described. Three or four replicates of each pair and control

Table 2. Strains of *Endothia parasitica* used as recipients of CH agents.

Strain	Designation	Isolate/type source	Geographical origin	Vegetative compatibility group
EP-42	BAR V	MI[a]	Connecticut	5
EP-144	7-8-1	MI	West Virginia	30
EP-155	p17+3	MI	Connecticut	40
EP-305	14A	MI	Connecticut	40
EP-366	p12-5	SAI[b]	Connecticut	39
EP-393	338x290,p7t2-3	SAI, cre,[c] met-[d]	Connecticut	5
EP-502	None	MI	Connecticut	10
EP-516	EP-6-b-4	SCI,[e] EP-6,met-	Connecticut	8
EP-523	60N	SCI,EP-60-b-9	Michigan	9
EP-589	234N	SCI,EP-234	Tennessee	24
EP-709	700N	SCI,EP-700	Virginia	49
EP-46	1012	MI	Tuscany, Italy	11
EP-62	None	SCI,EP-49	Tuscany, Italy	12
EP-65	None	SCI,EP-51	Tuscany, Italy	11
EP-67	None	SCI,EP-50	Tuscany, Italy	10
EP-408	2-4	MI	Tuscany, Italy	12
EP-409	3-8	MI	Tuscany, Italy	40
EP-421	22-8-b-6	SCI,22-8	Tuscany, Italy	11
EP-427	28-1	MI	Piedmont, Italy	20
EP-432	38-1	MI	Campania, Italy	30
EP-462	58-2	MI	Campania, Italy	30
EP-505	27-9-c-76	SCI,27-9	Piedmont, Italy	40

[a] Mass isolate.
[b] Single ascospore isolate.
[c] Cream mutant.
[d] Methionine-requiring mutant.
[e] Single conidial isolate.

were used in each experiment. Plates were sealed with parafilm and incubated under standard conditions.

Donor strains used in early transmission experiments included single conidial derivatives of source strains and a few products of earlier transmission experiments. New infection products were added to the list of donors as they were obtained. Recipient strains not successfully infected in early experiments were paired with the new donors as they became available. To check that transmission had occurred in cases of difficult transmission, products were paired with the uninfected form of the recipient strain. Rapid transmission between a product and its corresponding uninfected form was taken to indicate that the CH agent had been transmitted successfully.

Field pathogenicity test. Eight of the 22 sets of strains were tested for short term pathogenicity in eight 11 to 14 cm d.b.h. (diameter at 1.4 m) American chestnut sprouts in the forest. Each set included a recipient strain in its uninfected form and the same strain in each of five infection states. On each tree, an approximately 3 m length of trunk with smooth bark was divided into eight nearly equal regions. Sets of strains were assigned to positions (regions) on the eight trees according to an 8 x 8 Latin square design. Infection states within a strain set were arranged randomly within the region assigned to the set. Inoculations were made as described (Elliston 1978). Canker length and width were measured to the limits of infection or injury two months after inoculation. Canker areas were calculated using the formula

for an ellipse.

Results

Transmission experiments and cultural characteristics of products. Each of the six CH agents was successfully transmitted into each of the 22 recipient strains of the fungus. The culture collection numbers for the products are listed in Table 3. Each agent similarly affected cultural characteristics of all 22 recipient strains. Two representative sets of strains are shown in Figure 1. The most variable effects were observed with H_{I_1} infections. Subcultures of strains infected with this mixture of agents differed in pigmentation, amount of aerial mycelium, arrangement of pycnidia, and density and organization of leading mycelium.

Table 3. Culture collection numbers for products of transmission experiments

CH agent	Recipient strain										
	42	144	155	305	366	393	502	516	523	589	709
A. American Recipient Strains											
H_{M_1}	1025	843	844	845	833	832	836	552	544	715	839
H_{M_2}	1056	852	868	869	855	851	864	570	524	850	863
H_{T_2}	1060	894	905	906	878	902	892	600	629	653	910
H_{V_1}	1057	913	915	916	718	719	911	806	716	804	708
H_{I_1}	1058	776	779	781	848	818	803	923	395	820	823
H_{I_2}	1059	821	780	805	822	819	775	924	721	722	824
B. Italian Recipient Strains											
	46	62	65	67	408	409	421	427	432	462	505
H_{M_1}	837	791	838	788	793	846	786	847	795	796	799
H_{M_2}	857	859	858	865	860	870	811	808	853	854	871
H_{T_2}	886	893	887	885	901	907	810	809	895	877	908
H_{V_1}	790	792	912	789	794	798	717	801	914	797	800
H_{I_1}	761	762	763	765	767	812	757	771	773	849	769
H_{I_2}	759	760	764	766	768	816	758	772	922	813	770

Pathogenicity tests. The results of the pathogenicity test are summarized in Table 4. No statistically significant differences were found among areas of cankers caused by the eight strains in the uninfected state or when infected with agents H_{M1} or H_{I2}. With strains infected with agents H_{M2}, H_{T2}, and H_{I2}, a few statistically significant differences were found but no

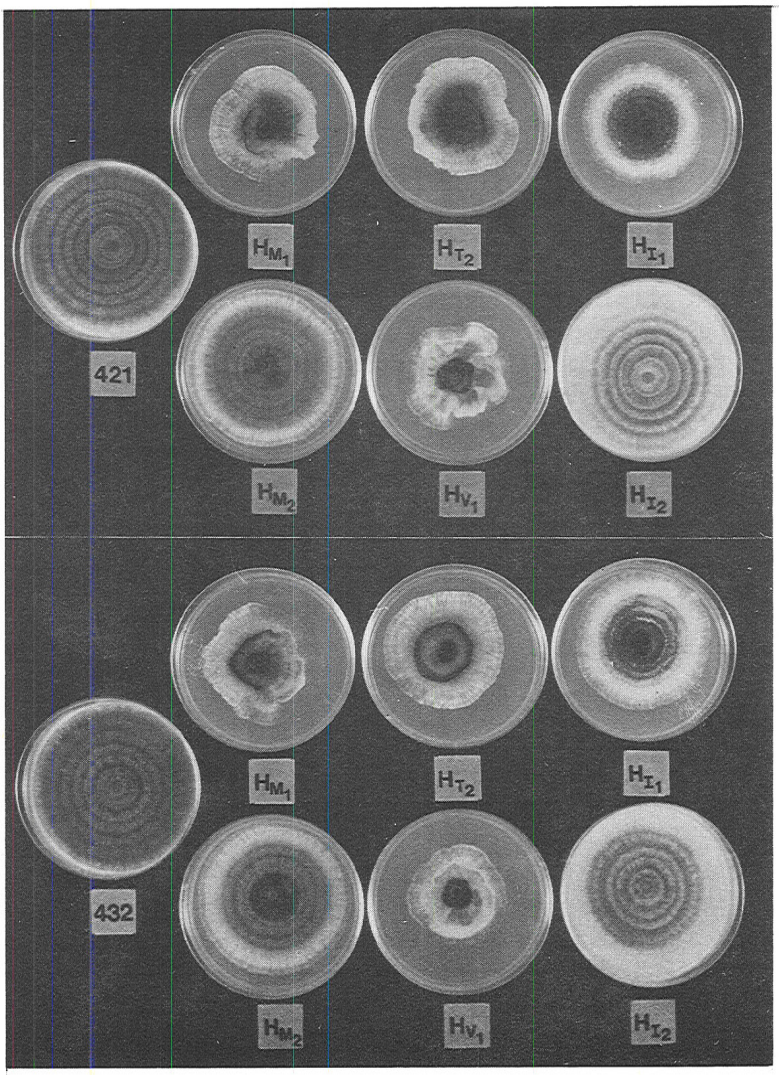


Figure 1. Representative recipient strains in their uninfected forms (left) and infected with six CH agents; A, recipient strain EP-421; B, recipient strain EP-432.

consistent patterns were observed. The statistical analysis of the data indicated highly significant differences occurred among trees (F=4,994), but not among inoculated regions within trees (F=0.21).

Discussion

In the study reported here, the five individual CH agents each had consistent effects on cultural characteristics of recipient strains whether the recipients were from North America or Italy or previously infected or not previously infected. Even EP-393, the cream mutant, showed consistent changes in colony morphology when infected with the agents; only colony color was distinct. Cultural characteristics of strains infected with H_{I_1}, the only mixture of agents used, were least consistent within subcultures of a given strain. This inconsistency may reflect the involvement of a mixture of agents. The inconsistency Anagnostakis and Day (1979) and Anagnostakis (1981) reported among derivatives of European strains may also have been caused by mixtures of agents.

Table 4. Mean canker area for eight recipient strains and their infection products

Recipient strain	Mean canker area, cm^2, for strain infected with CH agent: [y]					
	None	H_{M_1}	H_{M_2}	H_{T_2}	H_{I_1}	H_{I_2}
EP-144	54.0[z]	0.5	7.0^{bc}	0.8^{b}	16.8^{ab}	37.2
EP-155	58.6	0.8	11.4^{a}	1.6^{a}	18.1^{a}	42.6
EP-523	57.6	0.6	5.8^{c}	0.8^{b}	13.3^{abcd}	44.7
EP-589	56.8	1.0	8.4^{abc}	0.7^{b}	16.0^{abc}	52.0
EP-408	53.7	0.4	8.8^{abc}	0.8^{b}	12.4^{bcd}	51.3
EP-432	54.6	0.5	9.2^{ab}	1.5^{a}	9.9^{d}	42.2
EP-421	51.8	0.4	10.5^{a}	0.8^{b}	11.4^{cd}	45.7
EP-505	62.7	0.8	10.3^{ab}	2.0^{a}	13.7^{abcd}	42.4

[y] Means within columns not followed by the same letter are significantly different at $P \leq 0.05$, based on Duncan's "new multiple range test".

[z] Mean for eight cankers two months after inoculation.

The pathogenicities of the eight recipient strains in the uninfected form were not significantly different. This is in contrast with results reported previously by Jaynes and Elliston (1980) and may be due to having inoculated all strains into each tree in this experiment thus eliminating differences due to trees. Highly significant differences were found among trees in this study, but not among inoculated regions within trees. These results support the conclusion (Elliston 1978) that all strains in a pathogenicity determination should be inoculated into each tree, if that is feasible.

For the most part, a given CH agent affected the pathogenicities of the eight recipient strains similarly. The few significant differences found by statistical analysis may have been due more to the method of measuring cankers than to actual differences in amounts of colonized tissue. Measurements were made to the maximum limits of disrupted tissue. With weakly and moderately pathogenic strains, callus tissue often developed at the margin of the colonized tissue and sometimes caused uncolonized bark tissue at the top and bottom of the site to split and die back, enlarging the total amount of disrupted tissue and the calculated canker areas. The amount of callus was not uniform from site to site. The extent to which this affected the results is not clear.

Tha availability of many sets of recipient strains infected with a variety of CH agents will permit detailed comparisons of dsRNA patterns to determine if each agent has a consistently different pattern of dsRNA components. These strains will also be useful for establishing novel mixed infections and for preparing slurries (Jaynes and Elliston 1980) containing specific CH agents or combinations of agents for better defined studies of canker control.

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UTILIZATION OF CHESTNUT TANNINS BY ENDOTHIA PARASITICA

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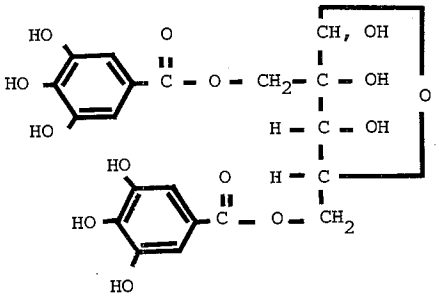
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ABSTRACT.--*As an extension of previous work on the utilization of hamamelitannin from the bark of blight-susceptible American and European chestnuts by Endothia parasitica, four other tannins were purified by semi-preparative high performance liquid chromatography and included, at a concentration comparable to that in the bark, as the only carbon source in a minimal medium inoculated with spores of E. parasitica. Two of the tannins, castalagin and vescalagin, had previously been shown to be present in susceptible and resistant (Chinese and Japanese) chestnuts. Two other unknown, late-eluting, and presumably higher molecular weight tannins from the bark and blight-resistant root bark of American chestnut were collected because of their high concentrations. The later eluting unknown tannin had a retention volume similar to that of a known procyanidin dimer of catechin and gave a colorimetric test for condensed tannins. All of the tannins were completely utilized by E. parasitica within four to ten days. Therefore it is concluded that chestnut tannins do not inhibit growth of E. parasitica but instead apparently support growth.*

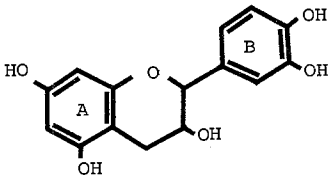
The utilization of tannins from the extracts of American chestnut *Castanea dentata* by the blight fungus *Endothia parasitica* was first observed by Cook and Wilson (1916). We have extended that work by first modifying the standard hide powder method for the analysis of tannins (Elkins and Wright 1977). The hide powder method is time consuming and requires large quantities of extracts to provide enough tannin to be weighed by difference upon adsorption onto hide powder (collagen). Our modification depends on the reduction of ultraviolet absorption following treatment of the extracts with polyamide (nylon) to remove the tannin. Polyamide worked better than other tannin-precipitating agents--hide powder, gelatin (soluble collagen), polyclar (cross-linked polyvinylpyrrolidone). Using the polyamide precipitation method, we have shown (Elkins, Pate, and Porterfield 1978) that *E. parasitica* utilizes tannins from the American chestnut, European chestnut *C. sativa*, and Chinese chestnut *C. mollisima*. There was greater utilization of tannins and greater mycelial weights of *E. parasitica* with the susceptible American chestnut and lesser utilization and mycelial weights with the resistant Chinese chestnut.

Nienstaedt (1953) and Hebard and Kaufman (1978) demonstrated a correlation between blight resistance and the qualitative differences in chestnut tannins. Extracts from the resistant Chinese chestnut contain only pyrogallol

tannins whereas extracts from the susceptible American and the resistant Japanese *C. crenata* chestnuts contain mixtures of catechol and pyrogallol tannins. Pyrogallol and catechol tannins are best described as hydrolyzable tannins and condensed tannins respectively. Hydrolyzable tannins such as hamamelitannin (1) (2-C-hydroxymethylribose) contain ester links which can be hydrolyzed by acid to simpler compounds (sugars and gallic acid). Their designation as pyrogallol tannins comes from the three adjacent phenolic OH's on gallic acid (as in pyrogallol). Condensed tannins are polymers of catechin (2) which further condense (polymerize) on treatment with acid. Their designation as catechol tannins comes from the two adjacent phenolic OH's on the B-ring of catechin (2) (as in catechol).



Hamamelitannin (1)



Catechin (2)

Nienstaedt's characterization of pyrogallol and catechol tannins depended on the reaction of each with formaldehyde and acid (Stiasny test). The activated center on the catechol tannins form gels (insoluble three-dimensional polymers) upon treatment with formaldehyde and acid whereas the deactivated center on pyrogallol tannins do not.

Condensed tannins are probably best characterized by the vanillin-sulfuric acid test (Ribereau-Gayon 1972) which gives a colorimetric reaction based on the interaction of vanillin with the highly activated phloroglucinol nucleus (A-ring) in catechin. We (Elkins and Lewis 1978) have used the vanillin-sulfuric acid test for condensed tannins in conjunction with the polyamide precipitation method for total tannins. The concentration of hydrolyzable tannins is determined by difference.

Our work has concentrated on the isolation of those specific tannin constituents which may either promote or inhibit the growth of *E. parasitica*. The tannin constituents are isolated from the lead tannins prepared by precipitation of the tannins from the extracts with basic lead acetate (Ribereau-Gayon 1972).

We (Elkins and Drumm 1980) have established a correlation between the presence of large quantities of hamamelitannin in the blight susceptible American and European chestnuts and its absence in the blight resistant Chinese and Japanese chestnuts. Two other complex hydrolyzable tannins, castalagin and vescalagin (Mayer 1971), were common to all four chestnut species. We have also shown that hamamelitannin is utilized by *E. parasitica* as a carbon source (Elkins, Graham, and Pate 1980). Thus it appears as if hamamelitannin promotes the growth of *E. parasitica*. Therefore, not only does *E. parasitica*

overcome any inhibitory effects of hamamelitannin, but it compounds the problem by putting the hamamelitannin to its own use.

Our recent work has focused on those high performance liquid chromatographic peaks found in the bark of large, surviving American chestnuts and in blight-resistant American chestnut root bark but absent from the bark of susceptible American chestnuts. Since one such unknown peak had previously been observed (Elkins and Drumm 1980), the chromatogram was expanded in the region of the unknown peak. A semi-preparative column was used to collect enough of each peak to inoculate with *E. parasitica*. The first two peaks were castalagin and vescalagin as determined by their retention volumes and ultraviolet spectra. The third peak was an unknown and the fourth peak was an unknown which gave a positive vanillin-sulfuric acid test for a condensed tannin. The retention volume for the unknown condensed tannin was in the same range as a known dimer of catechin, procyanidin B-2 (Thompson et al. 1972).

Castalagin, vescalagin, and the two unknown peaks were all rapidly utilized by *E. parasitica* (four to ten days) and supported growth of the fungus when they were the only carbon source in a minimal medium. Therefore we are forced to conclude that there is probably not an inhibitor to the growth of *E. parasitica* in the tannin fraction from large, surviving American chestnut trees.

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THE ABILITY OF HYPOVIRULENT ISOLATES AND MIXTURES OF HYPOVIRULENT ISOLATES TO CONTROL ARTIFICIALLY ESTABLISHED VIRULENT CANKERS

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ABSTRACT.--*The ability of hypovirulent (H) isolates of Endothia parasitica to alter the normal morphology of virulent (V) isolates was determined by individually pairing six H isolates and four H mixtures with V isolates from eight vegetative compatibility groups on agar media. The H isolates and H mixtures were scored as either compatible with a V isolate if morphological changes occurred, or incompatible if there was no change. The same V-H combinations (compatible and incompatible) were tested on American chestnut trees to determine whether the in vitro ability of an H isolate or H mixture to alter the morphology of a V isolate was similar to the in vivo ability to control canker expansion. With one exception, V isolates that were compatible in vitro with an H isolate or H mixture were always controlled in vivo. In all cases, H isolates and H mixtures controlled more V isolates in vivo than they morphologically altered in vitro.*

Introduction

The inability of vegetative hyphae to fuse has been reported for many ascomycetes, a few imperfect fungi, and for the chestnut blight fungus, *Endothia parasitica* (Anagnostakis 1978; Andes 1961; Caten 1972). When hyphae of two colonies of *E. parasitica* fail to anastomose on agar media, the result is the formation of a barrage, consisting of a ridge of asexual fruiting structures (Anagnostakis 1978; 1981b). This reaction has been termed vegetative incompatibility, and occurs commonly in North America among virulent (V) strains of *E. parasitica* (Anagnostakis 1981a; MacDonald and Double 1978). Genetic controls apparently govern the hyphal fusion and thereby determine vegetative compatibility (v-c) type (Anagnostakis 1981b; Day 1978). The restriction of fusion, due to vegetative incompatibility, may interfere with the transmission of hypovirulence determinants necessary for the conversion of a V strain to a hypovirulent (H) form (Anagnostakis 1978; Anagnostakis and Day 1979; Grente and Berthelay-Sauret 1978). The conversion of V strains to the H form occurs easily *in vitro* between strains of the same v-c type, but if the two strains are not of the same v-c type, conversion is sometimes slow and erratic, or may not occur (Anagnostakis and Day 1979).

The purpose of this study was to determine if V isolates, whether compatible

or incompatible *in vitro* with H isolates and H mixtures, were controlled *in vivo* when challenged with the same H isolates and mixtures.

Materials and Methods

The individual H isolates and the components of the H mixtures used in this study are listed in Table 1. Isolates were selected for each mixture, except the general, on the basis of their morphologic and pathogenic similarities (i.e. all components of the B mixture were white and nonpathogenic). The

Table 1. Components of the four hypovirulent mixtures

Designation	Components
B (nonpathogenic, white)	EP-14[a], EP-43[a], Grente's HV[b] 1-4-2w[c], 4-9-2w[c], 4-10-1w[c]
JR (nonpathogenic, pigmented)	JR 1[a], JR 2[a], JR 4[a], 4b7[a]
Italian (moderate pathogenicity and pigmentation)	EP-47[a], EP-48[a], EP-49[a], EP-50[a] EP-51[a]
General (varying pathogenicity and pigmentation)	All isolates listed above, plus EP-60[a], EP-90[a], EP-93[a], EP-102[a]

[a] Obtained from the Connecticut Agricultural Experiment Station, New Haven, CT 06504.

[b] Obtained from J. Grente, Clermont-Ferrand, France.

[c] Obtained from reisolations from natural cankers artificially inoculated with EP-43, West Virginia.

general mixture was comprised of all isolates in the B, JR, and Italian mixtures, plus four American components, EP-60, EP-90, EP-93, and EP-102. Inocula were prepared by first growing each of the 19 H isolates in three 250 ml Erlenmyer flasks containing 50 to 75 ml of semi-solid glucose yeast extract (GYE) agar, which consisted of 10.0 g of glucose, 2.0 g of Difco Bacto yeast extract, 1.0 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.2 mg of Fe++, 0.2 mg of Zn++, 0.1 mg of Mn++, 0.1 mg of thiamine hydrochloride, 0.01 mg of biotin, 4.0 g of Difco Bacto agar, and 1,000 ml of distilled water (Lilly and Barnett 1951). The flasks were then incubated at 25 C in a 16-hr photoperiod for 8 to 10 days and each flask was shaken vigorously after 3 days to enhance growth.

All 18 flasks of the six components comprising the B mixture were combined in a Waring blender and mixed at high speed for 45 to 60 seconds. Sterile solidified water agar (20 g/l) was added to 500 ml of this semi-solid mixture and blended until a loose gel consistency was obtained. The blended mixture was then transferred to 250 ml wash bottles for field use. The JR and Italian mixtures were prepared similarly, by combining their respective components.

The general mixture was prepared by combining equal amounts (approx. 100 ml) of the semi-solid B, JR, and Italian mixtures. The four American H components

were added to this mixture which was also thickened as above.

In vitro studies. Six H isolates and four H mixtures (Table 2) were paired separately against eight V isolates of different v-c types (A, B, C, D, E, F, G, and N) by the method described by Anagnostakis (1977; 1978). All tests were conducted by pairing mycelium plugs (2 to 3 mm square) of one V and one

Table 2. Virulent v-c types of *Endothia parasitica* that are converted *in vitro* and *in vivo* by individual hypovirulent (H) isolates and mixtures of H isolates

	v-c type converted		
		In vivo test	
Type	*In vitro* test[a/]	Controlled	Not controlled
Individual H Isolates			
EP-49	N	C, F, N	A, B
EP-50	N	C, F, N	A, B
JR 1	None	C, F, N	A, B
JR 4	N	C, F, N	A, B
1-4-2w	A	A, C, F	B, N
4-10-1w	B, F	C, F, N	A, B
H Mixtures			
B	A, B, D, E, F	A, B, C, D, E, F, G, N	-
JR	N	C, F, G, N	A, B, D, E
Italian	N	C, D, E, G, N	A, B, F
General	A, B, C, D, E, F, N	A, B, C, D, E, F, G, N	-

a/ Individual H isolates were tested *in vitro* against v-c types A, B, C, F, and N while the H mixtures were tested against v-c types A, B, C D, E, F, G, N.

H isolate on PDAmb (Difco potato dextrose agar supplemented with 0.1 mg/l of methionine and 0.01 mg/l of biotin) and incubated at 25 C in a 16-hr photoperiod (white fluorescent light) for 10 days (Anagnostakis 1977). Only one pair was tested per petri plate. Tests involving the mixtures were similar, however, in place of the H mycelium plug, a 0.5-cm diameter disk of agar was aseptically removed adjacent to the V plug and the resulting well was filled with the appropriate H mixture. All H and V combinations were replicated at least twice. Virulent isolates were scored as converted when a change in their morphology was observed (Anagnostakis and Day 1979).

In vivo studies. Virulent cankers of *E. parasitica* were artificially established on 39 American chestnut trees (*Castanea dentata*) in July 1978, for eventual challenge with H mixtures. Eight inoculations per stem, 15 to 20 cm apart, were made by removing a 0.5-cm diameter bark plug with a cork borer. A different V isolate from each v-c type (A, B, C, D, E, F, G, N) was inoculated into each wound by placing a similar size piece of agar containing V

mycelium into the inoculation site. These sites were covered with masking tape to retard drying. The V cankers were challenged 4 to 5 weeks after their initiation by punching 0.5-cm diameter bark plugs every 1 to 2 cm around the canker margin. The resulting holes were then filled with one of the four H mixtures and covered with masking tape. A similar treatment but with water agar was used as a check.

Twenty-seven additional American chestnut trees were inoculated with V isolates representing v-c types A, B, C, F, and N, for eventual challenge with H isolates. Each V isolate was introduced at two sites per stem. After 4 to 5 weeks, the resulting cankers were challenged with H isolates 1-4-2w, 4-10-1w; JR 1, JR 4; EP-49, and EP-50, selected from the B, JR, and Italian mixtures, respectively. These particular isolates were chosen according to their ability to convert certain v-c types *in vitro*. The V cankers were challenged as above, except an agar plug containing H mycelium was used in place of the H mixture. The length and width of each canker were measured at the time of the H challenge 3, 9, 15, 20, and 27 months after the challenge, and measurements were recorded either as identical in size at the time of the challenge or larger. Data are presented (Tables 3 and 4) as an average linear growth (cm) of each canker after 27 months and were determined using the formula $\frac{(L + W)}{2}$.

Table 3. Average linear growth (cm) of virulent v-c types of *Endothia parasitica* 27 months after challenged *in vivo* with four hypovirulent mixtures and water agar.

V-c type	Hypovirulent Mixtures y/				Water agar
	B	JR	Italian	General	
A	5.42b	10.89a	9.42a	4.25b	11.06a
B	4.75b	8.75a	10.42a	4.83b	9.67a
C	11.19b	11.92b	12.08b	5.83c	17.83a
D	5.25c	13.69a	9.19b	5.86c	15.33a
E	5.81c	10.14ab	8.67bc	5.92c	13.33a
F	6.31b	7.94b	11.92a	7.78b	15.33a
G	6.28c	9.17bc	12.31b	8.28c	19.83a
N	9.44b	6.75b	6.17b	8.00b	15.00a

y/ Average values followed by the same letter in each row do not differ significantly at P = 0.05.

Vegetative compatibility types were listed as either controlled, when the average linear growth of the challenged canker was significantly smaller than the water agar check, or not controlled when the significance values of the challenged canker and the check did not differ (Tables 3 and 4).

Results

Results of both the laboratory conversion *(in vitro)* test and the field challenge *(in vivo)* are in Table 2. The H mixtures were no better than the individual components comprising the mixtures when conversion tests with V

Table 4. Average linear growth (cm) of virulent v-c types of *Endothia parasitica* 27 months after challenged *in vivo* with six individual hypovirulent isolates and water agar

V-c type	Individual Hypovirulent Isolates[y]						Water agar
	EP-49	EP-50	JR 1	JR 4	1-4-2w	4-10-1w	
A	10.50ab	7.78bc	7.11bc	9.47abc	7.58bc	9.61abc	11.06a
B	14.72a	8.52b	8.25b	6.72b	7.97b	8.08b	9.67b
C	13.06b	10.86bc	11.05bc	10.72bc	7.97c	10.06bc	17.83a
F	11.89b	9.08bc	6.70c	7.08c	6.52c	6.72c	15.33a
N	9.61b	5.94c	9.05bc	6.02c	14.08a	8.38bc	15.00a

[y] Average values followed by the same letter in each row do not differ significantly at P = 0.05.

isolates of different v-c types were conducted *in vitro*. However, when the same V isolates were challenged in the field test, both the H mixtures and H isolates controlled more cankers than either did *in vitro*. For example, the Italian mixture, and two of the Italian components, EP-49 and EP-50, only converted v-c type N *in vitro*. Yet, the same mixture controlled the expansion of cankers incited by v-c types C, D, E, G, and N in the field test, and EP-49 and EP-50 controlled v-c types C, F, and N (Table 2). With one exception, all V isolates which were converted from the V to the H form *in vitro*, were also controlled in the field test by both individual H isolates and H mixtures. The exception was H isolate 4-10-1w which converted v-c type B in the laboratory, but did not control the canker expansion of v-c type B in the field.

Treatment with either the B or general mixtures resulted in significantly smaller cankers for each of the eight v-c types, when compared to cankers treated with water agar (Table 3). The JR and Italian mixtures only controlled canker expansion of four and five v-c types, respectively. No individual H isolate controlled canker expansion of all v-c types, but each of the individual H isolates was consistently better *in vivo* than *in vitro*.

Discussion

The chestnut blight fungus, *E. parasitica*, is commonly disseminated by ascospores (Anderson 1913; Anderson and Babcock 1913; Heald et al. 1915). Perithecia from a single wild-type canker frequently produce ascospores that are of different v-c type (Anagnostakis 1977). Consequently, ascospores that are infectious presumably would produce cankers that differ in v-c type. This seems to be the case for areas in North America where the v-c types of cankers have been determined (Anagnostakis 1981a; MacDonald and Double 1978). The diversity of v-c types found in the forest may significantly limit the transfer of cytoplasmic agents if the V and H strains do not interact because they are vegetatively incompatible. The findings of this study are encouraging because some v-c types that were not converted on agar media were controlled in the field test. For example, the B mixture converted v-c types A, B, D, E, and F in the laboratory, but controlled cankers incited by all v-c types challenged in the field.

Several reasons may exist for the more successful interaction of V and H isolates *in vivo*. One explanation may lie with the increased potential for anastomosis between V and H isolates afforded by the tree. The pattern of growth of *E. parasitica* within bark may provide greater opportunity for V and H mycelium to anastomose over a longer period of time than achieved in culture. Another explanation may involve the genetic controls that govern vegetative compatibility in *E. parasitica*. In an experiment where V and H strains were coinoculated in American chestnut trees, canker expansion was significantly limited when v-c types of the two strains differed at 0, 1, or 2 loci, but not significantly limited when the two strains differed at 5 or more loci (Anagnostakis and Waggoner 1981). Although the genetic relatedness among the V and H isolates used in this study is not known, the same genetic controls may be responsible for the successful *in vivo* control of combinations that were incompatible *in vitro*.

The term "control" was used in this study when the size increase of cankers treated with H inoculum was significantly less than cankers treated with water agar. The statistical tests undoubtedly were influenced by the method of canker measurement. In all cases, canker measurements were recorded either as identical to treatment size or larger. However, formation of callus sometimes resulted in the reduction in size of some cankers after treatment. If reduction in canker size had been considered, greater differences between the H and water agar treatments would have resulted, and the one V-H combination, v-c type B and H isolate 4-10-1w, that was successful in the laboratory may also have been successful in the field.

An additional problem was encountered with the virulent v-c type B isolate. The v-c type B cankers treated with EP-49 were significantly larger than the water agar checks. The increased canker size may not be due to the failure of EP-49 to control this particular v-c type, but might reflect the growth potential of this H isolate. Other workers have shown that EP-49 can colonize host tissue as readily as normal strains (Elliston 1978; Willey 1980). Table 4 illustrates that EP-49 treated cankers were almost always the largest of the H-treated cankers. Despite its high growth rate, EP-49 controlled as many cankers as the other five H isolates.

Data from this experiment has shown that individual H isolates and H mixtures more effectively control V isolates in a field situation, than suggested by their interaction *in vitro*. Vegetative compatibility may therefore not be as great a barrier to success of H as first thought. At this point, other factors such as method of establishing H inoculum, would seem to be a greater obstacle to success of H as a biological control.

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MYCELIUM PRODUCTION IN LIQUID MEDIA BY SEVERAL NORMAL, HYPOVIRULENT AND CONVERTED *ENDOTHIA PARASITICA* ISOLATES

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ABSTRACT.--*Tests were conducted with several liquid media to determine if mycelium production could be used to differentiate between normal, hypovirulent, and in vitro converted isolates of Endothia parasitica. Difco potato dextrose broth generally supported more mycelium formation than any other medium tested. Arginine supported the production of more mycelium than any other amino acid tested and more than the inorganic nitrogen source, ammonium tartrate. Cellobiose, glucose and fructose supported more mycelium formation in a defined medium with arginine as the nitrogen source than other carbon sources tested. Usually those hypovirulent isolates that grow faster and are white or slightly pigmented on Difco potato dextrose agar produced more mycelium than those hypovirulent isolates that grow slower and pigment more intensely. Mycelium production varied among normal isolates in each medium, and that produced by converted isolates often differed markedly from that produced by either of the original isolates. Mycelium production by four isolates with identical genetic backgrounds but with varying dsRNA components varied in some defined media. The normal isolates often produced more mycelium than the hypovirulent isolates, but mycelium production did not differentiate among the hypovirulent ones. Although mycelium production varied among the test media and among isolates, this cultural characteristic could not be used to reliably differentiate between the normal and hypovirulent isolates tested. Mycelium production on certain media may have been used for quantitatively differentiating those hypovirulent isolates that grow more rapidly and produce slight pigment from those that grow more slowly and pigment more intensely.*

Endothia parasitica, the fungus causing chestnut blight, nearly destroyed American chestnut as a forest tree in North America and caused severe losses to chestnut in Europe. Since the introduction of the fungus into North America in about 1904, many unsuccessful controls for the blight on American chestnut were tried, and interest in this problem waned. Recently however, there has been renewed interest in this disease. In Italy living blighted European chestnut trees were found with superficial healing cankers. Originally this was attributed to the development of resistance to *E. parasitica* in the host (Turchetti 1978). However, Grente and Berthelay-Sauret (1978a) recovered isolates of the chestnut blight fungus from these healing cankers that were less pathogenic than normal (N) ones, and possessed abnormal

cultural morphologies. When grown on a Maltea-Mosear agar, these hypovirulent (H) isolates often grew somewhat faster, sporulated less, and produced less pigment than N isolates (Grente and Berthelay-Sauret 1978b). By single sporing these isolates, an N and the slightly pigmented, white H isolate type were retrieved, as well as a slow growing pigmented type of H isolate, called JR (Grente and Berthelay-Sauret 1978b).

Hypovirulent isolates of *E. parasitica* also have been found in the United States (Day et al. 1977; Elliston 1978). These H isolates were obtained from American chestnuts that had survived the onslaught of chestnut blight and had cankers that were healing. The North American H isolates possess cultural morphologies on potato dextrose agar that differ from that of the N isolate types. Commonly they are pigmented but grow slower and sporulate less than N isolates.

To date, all H isolates tested contain dsRNA (Day et al. 1977; Elliston 1978) and its presence is thought to be responsible for the hypovirulence characteristics and the abnormal cultural morphologies. The nature and the mechanism of action of the dsRNA in H isolates, however, has not been fully characterized.

Workers have demonstrated (Anagnostakis 1978; MacDonald and Double 1978; Double personal communication) N and H isolates of *E. parasitica* can be readily differentiated when cultured on Difco potato dextrose agar (PDA). However these distinguishing criteria are subjective and dependent upon the cultural conditions used. Obviously the physiology of N and H isolate types must differ to account for the different cultural morphologies on PDA, but these differences cannot be adequately examined using media containing natural products. To date no studies on the specific physiological differences that exist between N and H isolate types using defined media have been reported. There are several reports, however, discussing the physiology of N isolates of this fungus.

Early reports indicated *E. parasitica* was capable of utilizing ammonium nitrogen and was partially deficient for thiamine and biotin (Lilly and Barnett 1951). Treggi (1954), using a Czapeks-Doxs medium, reported *E. parasitica* could utilize several amino acids as sources of nitrogen and if the concentration of glucose in this medium was increased, growth increased. Bazzigher (1958) reported that for this fungus to grow in a Knop-glucose solution, growth substances from chestnut bark were required. When the Knop-glucose medium contained bark extracts and thiamine, glutamic acid, aspartic acid, glutamine or asparagine were utilized effectively as nitrogen sources (Bazzigher 1958). He also showed biotin was required for growth on the inorganic nitrogen source, NH_4NO_3. Campbell (1967) reported the concentration of the carbon source (glucose or fructose) and the nitrogen source (asparagine) as well as the method of medium sterilization affected asexual sporulation by *E. parasitica*. Other work demonstrated a medium containing inorganic salts, glucose and thiamine supported good growth and asexual reproduction by this fungus, but when yeast and malt extract were added, growth and sporulation were enhanced (Puhalla and Anagnostakis 1971). Most recently, Uchida (1977) reported several *E. parasitica* isolates grew well in Richard's solution with glutamic acid, tyrosine, asparagine or methionine as nitrogen sources, but peptone supported optimum growth. He also reported the fungus grew well on a variety of mono-, di-, and

polysaccharides, but soluble starch was the best carbon source tested. With the resurgence of interest in the American chestnut blight problem and a general lack of information on the physiological differences that exist between N and H isolates, studies were initiated to determine: whether mycelium production in liquid media (dry weights) could be used to differentiate between N and H isolates of *E. parasitica;* whether medium constituents would differentially affect mycelium production among these isolates.

Materials and Methods

Sixteen isolates of *E. parasitica* were used in these tests: 16-15-1, an N isolate from West Virginia (grows somewhat slower on PDA than other N isolates); 6-3-1, an N isolate from West Virginia; 523, an N isolate derived from a single spore culture of H isolate 518 (Elliston 1981; 1982; personal communication); EP-49N, a single spore isolate of H isolate EP-49 with N culture morphology on PDA (Willey 1980); EP-43, a dsRNA containing H isolate (Day et al. 1977) that is fast growing and white or very slightly pigmented on PDA; EP-49, a dsRNA containing H isolate from Italy (Day et al. 1977) that is fast growing and slightly pigmented in PDA; EP-50, a dsRNA containing H isolate (Day et al. 1977) with cultural morphology quite similar to EP-49; 27-10, a dsRNA containing H isolate (Willey 1980) from Italy with cultural morphology on PDA similar to EP-49 and EP-50; EP-88, a dsRNA containing H isolate (Elliston 1978) that is slower growing and produces more pigment on PDA than EP-49, EP-50, or 27-10; EP-4, a dsRNA containing H isolate (Elliston 1978) that produces abundant pigment and grows slowly on PDA; 518, an H isolate from Michigan (Elliston 1978) that produces pigment, grows slowly on PDA and contains dsRNA components identified as Hm_1 and Hm_2 (Elliston 1981; 1982; personal communication); 524, an H isolate derived from a single spore of 518 that produces less pigment, grows faster on PDA than its parent isolate 518 and contains the dsRNA component identified as Hm_2 (Elliston 1981; 1982; personal communication); 544, an H isolate derived from a single spore of 518 that produces abundant pigment, grows somewhat faster on PDA than its parent isolate 518 and contains the dsRNA component identified as Hm_1 (Elliston 1981; 1982; personal communication); 16-15-1 plus EP-4, an *in vitro* convertant of N isolate 16-15-1 by H isolate EP-4; 16-15-1 plus EP-50, an *in vitro* convertant of N isolate 16-15-1 by H isolate EP-50; 6-3-1 plus EP-43, and *in vitro* convertant of N isolate 6-3-1 by H isolate EP-43.

All media tested, except Difco Bacto potato dextrose broth (PDB), contained a basic complement of constituents: 1.0 g KH_2PO_4, 0.5 g $MgSO_4$, 0.2 mg Fe^{++} ($FeSO_4 \cdot 7H_2O$), 0.2 mg Zn^{++} ($ZnSO_4 \cdot 7H_2O$), 0.1 mg Mn^{++} ($MnSO_4 \cdot H_2O$), 0.1 mg thiamine, and 1000 ml distilled water. The carbon and nitrogen sources tested, which varied according to the test, are presented in the results section. Except for media containing 10 g glucose and 2 g casein hydrolysate, 2 g peptone or 2 g yeast extract, all contained the amount of nitrogen provided by 2 g asparagine and the amount of carbon provided by 10 g glucose. The pH of all test media except those containing natural products was adjusted to 6.0 ± 0.1 with KOH or H_2SO_4. After pH adjustment, all media were dispensed into 250 ml Erlenmeyer flasks (25 ml/flask) and autoclaved. Agar plug inocula were placed in the flasks, one plug per flask with five replicate flasks of each medium per isolate. The resulting cultures were incubated at 25 C for 10 days in diffuse 12/12 light and dark. The resulting mycelium was harvested through a fine cloth, dried at 80 C and weighed to the nearest mg.

Analyses of variance were conducted and F-values calculated on mycelium production in each medium to determine if significant differences in mycelium production existed among isolates. In addition, Duncan's multiple range tests at 5 percent were conducted on mycelium production among media to determine which medium supported most mycelium production, and among isolates to determine if they could be separated into N or H isolate types using mycelium production (Steele and Torrie 1960; Snedecor and Cochran 1967).

Results and Discussion

Potato dextrose broth supported more overall mycelium production than all other media tested (Tables 1, 2, 3 and 4). Of the other media containing natural products (Table 1), the medium containing yeast extract as the nitrogen source supported less mycelium production than PDB, but more than the media containing casein hydrolysate or peptone as nitrogen sources (Table 1). The optimal mycelium production in PDB is not surprising. Difco PDA, a medium similar to PDB but containing Difco Bacto agar, also supports good growth of *E. parasitica* and is the medium of choice for growth of this fungus by many workers. In fact, the cultural characteristics produced on PDA serve as one *in vitro* standard for differentiating between N and H isolate types. The relatively poor performance of peptone in our test is at variance with those reported by Uchida (1977). Peptone generally is considered to be a good nitrogen source for fungi including *E. parasitica* (Shear et al. 1917), but in our tests, supported no more mycelium production than some of the amino acids. The differences in cultural conditions we used, compared to those of Uchida (1977) and Shear et al. (1917) might account for the difference in the results with peptone.

Table 1. Mean values for mycelium production (mg) among 16 isolates of *Endothia parasitica* grown for 10 days at 25 C in 25 ml of liquid media containing natural products

Medium	Dry weights[y/]
Potato Dextrose Broth	144a
Yeast Extract	121b
Casein Hydrolysate	89c
Peptone	75c

y/ Different letters in column indicate means significantly different at the 5 percent level using the Duncan's multiple range test.

Table 2. Mean values for mycelium production (mg) by 16 isolates of *Endothia parasitica* grown for 10 days at 25 C in 25 ml of a defined liquid medium containing glucose as the carbon source and one of six nitrogen sources

Nitrogen source	Dry weights[y/]
Arginine	82a
Asparagine	60b
Proline	52bc
Valine	41cd
Methionine	32d
NH_4 Tartrate	32d

[y/] Different letters in column indicate means significantly different at the 5 percent level using the Duncan's multiple range test.

Table 3. Mean values for mycelium production (mg) by 16 isolates of *Endothia Parasitica* grown for 10 days at 25 C in 25 ml of a defined liquid medium containing fructose as the carbon source and one of five nitrogen sources

Nitrogen source	Dry weights[y/]
Arginine	76a
Proline	44b
Valine	41b
Methionine	29c
NH_4 Tartrate	21d

[y/] Different letters in column indicate means significantly different at the 5 percent level using the Duncan's multiple range test.

Even though the mean value for mycelium production in PDB was 144 mg, the amount of mycelium produced by the individual isolates ranged from a high of 281 mg for isolate EP-49 to a low of 58 mg for isolate EP-4 (Table 5). With the exception of EP-43, those H isolates (EP-49, EP-50 and 27-10) that grow more rapidly and produce limited pigment on PDA produced more mycelium than those H isolates (EP-4, EP-518, EP-524 and EP-544) that grow more slowly on PDA and pigment more intensely. These results are not surprising when the relative amounts of radial growth on PDA by these isolates are compared. Mycelium production also varied significantly among N isolates, and 16-15-1, which grows somewhat slower on PDA than some other N isolates, produced less mycelium than two of the other N isolates tested, 6-3-1 and EP-49N. Converted isolate 16-15-1 plus EP-4 produced more mycelium than its original isolates and more than convertant 16-15-1 plus EP-50. Mycelium production did not discriminate between the four isolates (EP-518, EP-523, EP-524 and EP-544) with identical genetic backgrounds but with varying dsRNA components.

Potato dextrose agar is routinely used to discriminate between the isolates of *E. parasitica* containing these components, but the criteria Elliston (1981 this proceedings) uses to distinguish between isolates containing these dsRNA components, including radial growth on PDA, do not seem to be associated with mycelium formation in PDB (Table 5).

Table 4. Mean values for mycelium production (mg) by 16 isolates of *Endothia parasitica* grown for 10 days at 25 C in 25 ml of a defined liquid medium containing arginine as the nitrogen source and one of five carbon sources

Carbon source	Dry weights[y]
Cellobiose	86a
Glucose	82a
Fructose	76ab
Pectin	64b
Soluble starch	37c

[y] Different letters in column indicate means significantly different at the 5 percent level using the Duncan's multiple range test.

Table 5. Mycelium production (mg) by 16 isolates of *Endothia parasitica* grown for 10 days at 25 C in 25 ml of potato dextrose broth

Isolates[y]	Dry weights[z]	Isolates[y]	Dry weights[z]
H EP-49	281a	C 6-3-1 + EP-43	135cde
C 16-15-1 + EP-4	253a	H 518	97def
H EP-50	237ab	H 524	97def
C 16-15-1 + EP-50	192bc	N 16-15-1	84ef
H EP-88	186bc	H 544	83ef
H 27-10	170c	N 523	78ef
N 6-3-1	155cd	H EP-43	63f
N EP-49N	139cd	H EP-4	58f

[y] H, dsRNA containing hypovirulent isolates; C, converted isolate; N, normal virulent isolate.

[z] Different letters in column indicate means significantly different at the 5 percent level using the Duncan's multiple range test.

Arginine supported more mycelium production in the media containing glucose or fructose as carbon sources than the other amino acids tested and more than the inorganic nitrogen source, ammonium tartrate (Tables 2 and 3). Methionine and ammonium tartrate supported the least mycelium production

whereas intermediate amounts of mycelium were produced in the glucose-asparagine, -proline, and -valine media and in the fructose-proline and -valine media (Tables 2 and 3). These results are at variance with those of other workers. Asparagine (Bazzigher 1958; Uchida 1977) and methionine (Uchida 1977) were reported to support optimum growth by this fungus.

Cellobiose and glucose supported more mycelium formation in the medium containing arginine as the nitrogen source than pectin or soluble starch (Table 4). Fructose supported mycelium production in this medium equal to that produced in the cellobiose, glucose and pectin media (Table 4). Uchida (1977) reported *E. parasitica* grew in media with a variety of mono-, di- and polysaccharides as carbon sources, but soluble starch supported optimum growth. This carbon source supported the least mycelium production of the carbon sources we tested (Table 4). Pectin and cellobiose have not been tested as carbon sources by other workers, but since these materials are components of plant cell walls, they probably serve as carbon sources for this fungus growing in chestnut bark. With the inability of some H isolates (EP-43 and EP-4) (Double personal communication) to develop cankers in American chestnut, it was thought the inability of these isolates to establish a food relationship with the host might be associated with the lack of canker development. Our data do not eliminate this as a basis for the lack of canker formation, but since these isolates can use cellobiose and pectin as carbon sources the lack of a carbon source probably is not involved with their inability to form cankers.

Mycelium production in the glucose-, fructose- and cellobiose-arginine media also varied among isolates (Tables 6, 7 and 9). Those H isolates that grow more rapidly and are white or only slightly pigmented on PDA (EP-43, EP-49, EP-50 and 27-10), produced more mycelium in these media than those H isolates but grow more slowly and pigment more intensely (EP-4, EP-518, EP-523 and EP-524). This is the same type of difference noted in PDB (Table 5). Mycelium production among N isolates on these media also varied (Tables 6 and 7), similar to that observed in PDB. However, the amount of mycelium produced by the individual N isolates in the test media was not consistent. Normal isolates 16-15-1 produced less mycelium than 6-3-1 or EP-49N in PDB, but equal to or more than those isolates in the glucose- and fructose-arginine media. Mycelium production by converted isolates in the glucose-, fructose-, and cellobiose-arginine media often differed from each other and from that of the original isolates. The mycelium production of the four isolates with identical genetic backgrounds with varying dsRNA components differed on these media. The N isolate, EP-523, consistently produced more mycelium than the H isolates, EP-518, EP-524, and EP-544, but mycelium production did not discriminate among the H isolates.

Although less mycelium was produced in the glucose-valine medium than in any of the other media presented in this report, many of the same general mycelium production patterns present in the other media, were evident (Tables 5, 6, 7, 8 and 9). The H isolates that grow more rapidly and are white or slightly pigmented on PDA (EP-43, EP-49, EP-50 and 27-10) produced more mycelium than those H isolates (EP-4, EP-518, EP-524 and EP-544) that grow more slowly and pigment more intensely. There was less variation in mycelium production among N isolates in this medium than in the others, and mycelium production by N isolate EP-523 only differed from that produced by H isolates EP-518 and EP-524. Mycelium formation by the converted isolates often differed from one another and from one or both of the original isolates.

Table 6. Mycelium production (mg) by 16 isolates of *Endothia parasitica* grown for 10 days at 25 C in 25 ml of a defined liquid medium containing glucose as carbon source and arginine as the nitrogen source

Isolates[y]	Dry weights[z]	Isolates[y]	Dry weights[z]
H 27-10	182a	C 16-15-1 + EP-4	70fg
H EP-49	139b	N EP-49N	67fgh
H EP-50	127bc	C 16-15-1 + EP-50	62gh
H EP-43	122bcd	H EP-88	60gh
C 6-3-1 + EP-43	104cde	N 6-3-1	50gh
N 523	95def	H 524	39hi
N 16-15-1	77efg	H 544	21ij
H EP-4	72fg	H 518	11j

[y] H, dsRNA containing hypovirulent isolates; C, converted isolate; N, normal virulent isolate.

[z] Different letters in column indicate means significantly different at the 5 percent level using the Duncan's multiple range test.

Table 7. Mycelium production (mg) by 16 isolates of *Endothia parasitica* grown for 10 days at 25 C in 25 ml of a defined liquid medium containing fructose as the carbon source and arginine as the nitrogen source

Isolates[y]	Dry weights[z]	Isolates[y]	Dry weights[z]
H EP-50	148a	N 6-3-1	58g
H 27-10	139ab	H EP-88	57gh
H EP-49	126bc	N 16-15-1	54gh
H EP-43	112cd	H EP-4	48gh
C 16-15-1 + EP-4	102de	C 6-3-1 + EP-43	43h
N EP-49N	89ef	H 524	25i
N 523	87ef	H 544	22i
C 16-15-1 + EP-50	85f	H 518	18i

[y] H, dsRNA containing hypovirulent isolates; C, converted isolate; N, normal virulent isolate.

[z] Different letters in column indicate means significantly different at the 5 percent level using the Duncan's multiple range test.

Although these nutritional studies were not exhaustive, only 20 media were tested, and only a small number of *E. parasitica* isolates used, it is obvious the nutritional constituents of liquid media affected mycelium production (Tables 1, 2, 3 and 4). Potato dextrose broth generally supported more mycelium production than all other media tested, and glucose was equal to or better than the other carbon sources tested in the defined media. The amino

acid arginine was the nitrogen source that supported optimal mycelium production in the defined media.

Although detailed results are presented for only 5 of the 20 media tested, the variation among isolates in media not mentioned in this report were similar to that in the medium specifically mentioned (Tables 5, 6, 7, 8 and 9). Often those H isolates that grow most rapidly and are white or slightly pigmented on PDA produced more mycelium than those H isolates that grow more slowly and pigment more intensely. The mycelium production of the N isolates and H isolate EP-88 often was somewhat intermediate to these two H isolate types, but due to the variation in mycelium formation that existed among the N and H isolates, it was not possible to use mycelium formation in any of the liquid media tested as a quantitative means to discriminate between these isolate types.

Table 8. Mycelium production (mg) by 16 isolates of *Endothia parasitica* grown for 10 days at 25 C in 25 ml of a defined liquid medium containing glucose as the carbon source and valine as the nitrogen source

Isolates[y/]	Dry weights[z/]	Isolates[y/]	Dry weights[z/]
H 27-10	143a	C 16-15-1 + EP-50	32ef
H EP-49	71b	H 544	30ef
H EP-88	61bc	N 523	26fg
H EP-43	54cd	C 16-15-1 + EP-4	26fg
H EP-50	53cd	C 6-3-1 + EP-43	16g
N 16-15-1	41de	H 524	11hi
N EP-49N	38ef	H 518	7hi
N 6-3-1	32ef	H EP-4	3i

[y/] H, dsRNA containing hypovirulent isolates; C, converted isolate; N, normal virulent isolate.

[z/] Different letters in column indicate means significantly different at the 5 percent level using the Duncan's multiple range test.

Our data also did not demonstrate nutritional constituents in the test media differentially affected mycelium production among these isolates. The reduced mycelium production by H isolate EP-88 in some of the defined media compared to that in PDB (Tables 5, 6, 7, 8 and 9) and the relatively good mycelium production by H isolates EP-4 in the glucose-arginine medium (Table 6), compared to that in the glucose-valine medium (Table 9), suggest differential affects may exist, but more tests must be conducted to determine if these differences are consistent.

The variation in mycelium production that often existed between the converted isolates and either of their original isolates demonstrates an interaction must exist between the N isolates that is being converted and the specific dsRNA component(s) of the H isolate doing the converting. Converted isolate 16-15-1 plus EP-4, for example, produced more mycelium in PDB than its original isolates and more than convertant 16-15-1 plus EP-50. The two

converting isolates, EP-4 plus EP-50, produced very different amounts of mycelium in PDB, but when they were used to convert this isolate, 16-15-1, the resulting convertants produced mycelium quite different from one another and from that of either of the original isolates (Table 5). Similar differences in mycelium formation by these convertants were evident in other test media (Tables 6, 7, 8 and 9).

Table 9. Mycelium production (mg) by 16 isolates of *Endothia parasitica* grown for 10 days at 25 C in 25 ml of a defined liquid medium containing cellobiose as the carbon source and asparagine as the nitrogen source

Isolates[y]	Dry weights[z]	Isolates[y]	Dry weights[z]
H 27-10	125a	C 16-15-1 + EP-50	93cd
H EP-49	122ab	N 16-15-1	92cd
H EP-43	117abc	C 6-3-1 + EP-43	89de
N 523	116abc	H EP-88	89de
N EP-49N	102abc	H 524	64e
N 6-3-1	97bcd	H 518	30f
H EP-50	97bcd	H 544	24f
C 16-15-1 + EP-4	96bcd	H EP-4	19f

[y] H, dsRNA containing hypovirulent isolates; C, converted isolate; N, normal virulent isolate.

[z] Different letters in column indicate means significantly different at the 5 percent level using the Duncan's multiple range test.

Elliston (1981 this proceedings; personal communication) suggests H isolates EP-518, EP-524, and EP-544 only differ in their dsRNA components, and the presence of these specific dsRNA's confers distinct cultural and pathogenic abnormalities to these isolates. It was unfortunate mycelium production would not discriminate between these isolates. It was hoped mycelium formation might serve as a tool for quantitative differentiation.

With the almost total lack of information on physiological differences that exist between N and H isolate types as well as within various H types, the results of this study can only serve as a starting point for additional tests. Such physiological studies should continue because results should provide a more complete understanding of the basis for the cultural and pathological abnormalities conferred on *E. parasitica* by the hypovirulence factor(s).

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ISOLATION AND PARTIAL CHARACTERIZATION OF A VIRUS-LIKE PARTICLE AND ITS GENOME ASSOCIATED WITH ENDOTHIA PARASITICA STRAIN 43

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ABSTRACT.--*A virus-like particle (VLP) infecting Endothia parasitica strain 43 has been partially purified by polyethylene glycol (PEG) precipitation, differential centrifugation and isopycnic density gradient centrifugation in cesium chloride (CsCl) and cesium sulphate (Cs_2SO_4). The buoyant density of the particle was determined to be 1.207 and 1.177 g/cc respectively. A sedimentation (S) value of 164 was calculated for the particle. Examined under the electron microscope, the VLP's were pleiomorphic in morphology with a diameter of 100 nm. The particles possessed an ultraviolet absorption profile characteristic of nucleoproteins. The viral genome was extracted, partially purified and found to consist of two segments of double-stranded ribonucleic acid (dsRNA). The molecular weights of the two components were resolved by polyacrylamide gel electrophoresis to be 6.76 and 6.02 x 10^6 daltons. The dsRNA possessed an S value of 34, a buoyant density of 1.597 g/cc in Cs_2SO_4, and upon thermal denaturation a 30 percent shift in absorbance was observed with a Tm value of 89.3 C. Partially purified preparations from a virulent strain lacked the VLP's and any detectable dsRNA.*

Since the first report of a virus in a fungus (Gandy and Hollings 1962), viruses have been observed in over 100 fungal species and in some 60 genera of fungi (Saksena and Lemke 1976). Due to the unusual characteristics and nature of the hypovirulent strains of *Endothia parasitica*, the cause of chestnut blight, the possibility of involvement of a virus was investigated.

To examine for the presence of a fungal virus, two different strains of *E. parasitica* were tested. One was a hypovirulent strain, 43; the other an American virulent, strain 671-b, which served as a control. The cultures were maintained on Difco potato dextrose agar and grown in liquid glucose-yeast extract (GYE) for subsequent virus extractions.

A modified method (Dodds 1978) was developed to allow the quick and simple isolation of the virus-like particles (VLP's). Fifty to 100 grams of freshly harvested mycelia were homogenized in 0.1 M sodium acetate buffer, pH 5.0. Following centrifugation, the supernatant was adjusted to 10 percent PEG-6000 and 0.3 M sodium chloride and incubated at 4 C. The VLP's were pelleted and subjected to two rounds of differential centrifugation, followed by a final purification by isopycnic density gradient centrifugation.

The partially purified and the purified VLP's showed a typical nucleoprotein profile with peaks at 260 and 280 and unusual peaks at 293, and 269 nm. These same absorbancy peaks are not observed when the virulent strain was tested in the same experiment.

Virus-like particles were observed to be somewhat pleiomorphic in morphology when negatively stained in 2 percent phosphotungstic acid (PTA) and examined under the electron microscope. The particles had a diameter of 100 nm (Figure 1) and closely resembled the virus associated with a disease of cultivated mushrooms (Lesemann and Koenig 1977). No virus particles were detected in preparations from the virulent strain. To examine the possibility of the particle being membrane bound, the VLP's were treated with chloroform and reexamined under the electron microscope. No particles were observed suggesting the presence of a lipid membrane, possibly involved as an integral part of the virus structure.

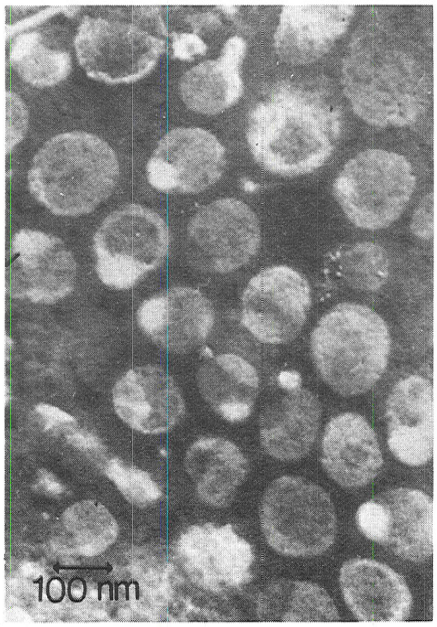


Figure 1. Electron micrographs of the partially purified VLP's stained with 2 percent PTA. Magnification of the particles is 165,000x and the bar represents 100 nm.

The buoyant density of the particle was determined by banding in cesium chloride (CsCl) and cesium sulphate (Cs_2SO_4). The particles banded at 1.207 and 1.177 g/cc respectively. To determine the sedimentation value, the virus particle was banded in a linear 5 percent to 50 percent sucrose gradient and with Squash Mosaic Virus 118 S, 95 S and 57 S RNA as markers. A value of 164 S was determined. The low density and the pleiomorphic appearance of the particle supports the contention that the particle is lipid bound.

The nucleic acid of the particle was extracted by a single phase phenol-chloroform-sodium dodecyl sulfate (SDS) treatment. The nucleic acid had a typical uv absorption profile with a maxima at 260 nm and a minima at 236 nm.

The nucleic acid was negative with diphenylamine reaction, and was positive with orcinol and was resolved into two major segments by polyacrylamide gel electrophoresis (Figure 2, lanes C and D). These segments were resistant to RNAase in high salt concentration and to DNAase, but were sensitive to RNAase in low salt. Upon thermal denaturation, a 30 percent hyperchromic shift was observed indicating that the nucleic acid of the particle is dsRNA.

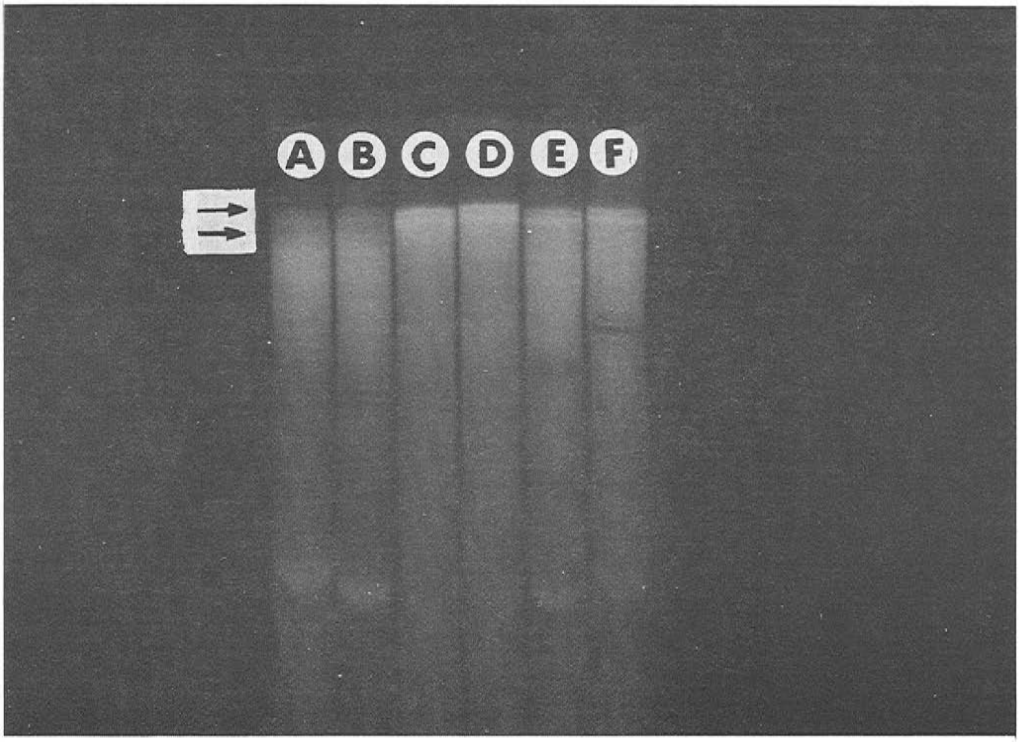


Figure 2. Polyacrylamide gel electrophoresis of the dsRNA extracted from the fungal mycelia, lanes A and B; from the viral particle, lanes C and D; both sources of dsRNA coelectrophoresed, lanes E and F. Note the same banding pattern obtained.

The dsRNA was extracted from the fungal mycelium of strain 43 directly. Two different extractions protocols were tested in the isolation procedure. In the first protocol, a modified method (Franklin 1966) of a phenol-SDS extraction was used, followed by Whatman CF-11 column chromatography. The isolated dsRNA demonstrated a typical uv absorption for nucleic acids and a 260/280 ratio of greater than 1.80 was consistently obtained. Total yield of the dsRNA was relatively low ranging between 1.2 to 1.6 µg/g of tissue.

The second isolation procedure (Morris and Dodds 1979) was modified to increase the dsRNA yield from strain 43. The method employed the direct addition of CF-11 cellulose (treated with tRNA) to the nucleic acid solution. The dsRNA was eluted and repassed through a second column of CF-11 cellulose. Significantly higher yields of dsRNA were obtained in the range of 4.8 to 5.4 µg/g of fungal tissue.

DsRNA isolated by either protocol was resolved by gel electrophoresis into two major components (Figure 2, lanes A and B). Molecular weight estimations of the isolated dsRNA were based on the migration patterns of other dsRNA species with known molecular weights and determined to be 6.76 and 6.02 x 10^6 daltons. The dsRNA was observed to be resistant to DNAase and RNAase in high salt while sensitive to RNAase in low salt. The dsRNA's extracted from the virus particle and the fungal mycelia were coelectrophoresed (Figure 2, lanes E and F) and were observed to have the identical banding patterns indicating the dsRNA isolated from either the particle or the mycelia are the same. Both sources of dsRNA demonstrated a buoyant density of 1.597 g/cc in Cs_2SO_4, again a good indication of the double-stranded nature of the RNA, and had an S value of 34. The dsRNA isolated from the fungal mycelia was heat denatured and a 30 percent increase in the absorbance was detected with a Tm value of 89.3 C. These results are consistent with the results of the dsRNA extracted from the particle and clearly indicate the RNA was double-stranded.

Conclusions

The VLP isolated from *E. parasitica* strain 43 could be classified as a mycovirus; the particle possesses a genome of dsRNA, having definite size and shape. This report marks the first instance of a VLP infecting *E. parasitica* to be termed as a mycovirus.

The dsRNA extracted from the mycovirus and from the fungal mycelia are exactly the same. Experiments using gel electrophoresis, nucleases, molecular weight estimations, density gradient centrifugation and thermal denaturation confirmed this nature. The dsRNA associated with the hypovirulent strains of *E. parasitica* has been assigned the responsibility as the factors determining the hypovirulence nature of these strains.

The dsRNA components can be isolated in a particle that is lipid bound. The particle was isolates intact and can be referred to as a virus-like particle or a mycovirus. The work presented that the true nature of the hypovirulence phenomenon may reside in this virus particle.

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LIPOSOME ENCAPSULATED DSRNA FOR CELL-FREE TRANSMISSION OF HYPOVIRULENCE

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ABSTRACT.--*Unilamellar lipid vesicles prepared by the ether injection method were used to capture dsRNA prepared from Endothia parasitica strain EP-113. The liposomes were fused with protoplasts of an E. parasitica virulent, methionine auxotrophic strain (EP-6). Protoplasts were regenerated on potato dextrose agar containing 0.55 M salt. Cell-free infection of protoplasts was also attempted using free dsRNA and dsRNA containing membrane-bound particles. Presence of the white phenotype was initially used as an indication of cell-free transmission. However, conversion experiments using EP-34 (arg$^-$) indicated otherwise and attempts to extract dsRNA from the white colonies were unsuccessful. The relatively high percentage of white colonies probably resulted from osmotic shock.*

The evidence for dsRNA involvement as the carrier of genetic information of transmissible hypovirulence (TH) is based entirely upon correlative data (Elliston in press). While this correlative evidence is strong enough to justify research on the biology of the dsRNA, direct evidence of dsRNA involvement by cell-free transmission of the dsRNA is still needed. Development of a successful system for cell-free transmission of dsRNA will make possible studies such as synchronous infection, mixed infections, single segment infections and introduction of the dsRNA into new strains without mixing cytoplasmic genes.

Cell-free transmission of mycoviruses has proven to be very difficult. Relatively few reports exist of successful transmission of mycoviruses (Ghabrial 1980). There are no reports of transmission of dsRNA to fungi without the viral capsid and other associated peptides (Buck 1980). Thus, the task of cell-free transmission of the TH associated dsRNA will not be easy. To increase the likelihood for transmission, the dsRNA will be packaged within membrane vesicles. These vesicles can then be fused with fungal protoplasts releasing the vesicle contents into the protoplasts. Two different types of membrane vesicles for fusion with the fungal protoplast can be used: 1) the naturally occurring membrane-bound particles containing dsRNA that have been isolated from *Endothia parasitica* strain EP-113, and 2) artificially prepared liposomes that contain the dsRNA. While we have not yet successfully transmitted the dsRNA into virulent *E. parasitica* using either of these procedures, we have worked out most of the necessary details. The procedures for obtaining and regenerating protoplasts of virulent *E. parasitica* and the capture of dsRNA in liposomes is described.

Materials and Methods

Protoplasts of *E. parasitica* strain EP-6 are obtained by incubating approximately 0.3 ml of packed mycelium in the following protoplasting solution: 2.7 ml 5 mM phosphate buffer, pH 5.5 containing 0.55 M NaCl, 45 mg Sigma cellulase, and 0.3 ml of Sigma β-glucuronidase. The mycelium is prepared by inoculating flasks of *E. parasitica* complete medium (Puhalla and Anagnostakis 1971) with conidia and incubating with shaking. Mycelium is harvested by removing the desired amount and washing by centrifugation twice with 20 volumes of 5 mM phosphate buffer, pH 5.5 followed by one wash with 5 mM phosphate buffer with 0.55 M NaCl. The mycelium is incubated with the protoplasting solution at 25 C in a 50 ml Erlenmeyer flask shaking at 150 rpm for 4 to 8 hrs. The protoplasts are separated from mycelial debris by very low speed (250 rpm) centrifugation for 1 minute. The supernatant containing the protoplasts is pelleted by centrifuging at 1000 rpm for 3 minutes. Protoplasts are resuspended in 0.7 M NaCl. Protoplasts were regenerated by plating onto Inolex potato dextrose agar supplemented with 7.5 g/l Difco malt extract, 2.5 g/l Difco yeast extract, 0.1 g/l methionine and 0.55 M NaCl.

Liposomes were prepared using the ether injection method (Ostro et al. 1977). The procedure of Matthews et al. (1979) was used as the primary guide. The liposomes were prepared from an 8:2 molar ratio of phosphatidyl choline and dicetyl-phosphate. The lipids were dissolved in chloroform:petroleum ether (1:10) to a concentration of 4 μmole/ml. Five ml of lipid phase were injected into 1 ml aqueous phase at a rate of 0.5 ml/min. The aqueous phase consisted of dsRNA dissolved in 5 mM HEPES buffer, pH 7.4 with 0.15 M NaCl and 1 mM EDTA. After ether injection, any residual ether was removed by bubbling nitrogen through the liposome suspension.

Preparation of dsRNA and the dsRNA containing membrane-bound particles from *E. parasitica* strain EP-113 were as reported by Dodds (1980a; 1980b).

Results

Protoplast release and regeneration. The release of protoplasts from the mycelium occurs not by total dissolution of the cell wall but rather by extrusion of the protoplast from weakened portions of the wall. One of the first visible effects of the enzyme is the breaking apart of the mycelial cells at the cross-walls. Later protoplasts are seen extruding from these weakened walls or from hyphal tips. The particular combination of enzymes that we use appears to cause extensive release of protoplasts from cells. Some bursting of protoplasts occurs but shaking in an Erlenmeyer flask rather than a test tube reduces the number of burst protoplasts. Osmotica other than NaCl can be used. We tried 0.5 M mannitol and found no difference in survival rate. However, regeneration of protoplasts on mannitol resulted in colonies that were large and diffuse as opposed to the desired compact colonies on NaCl agar.

We tried a number of methods to separate the protoplasts from the mycelial cell ghosts. Because they both pellet upon centrifugation at about the same rate, even through dense solutions, separation is difficult. The mycelial ghosts do pellet faster at very low speed (250 rpm) than do protoplasts.

Using this procedure most of the protoplasts remain suspended. The protoplast suspension is contaminated with 0.1 to 2 percent colony-forming propagules that do not burst when diluted with water.

Yield of protoplasts from the approximate 0.3 ml packed volume of mycelium was about 10^4 regenerated colonies/ml. Efficiency of regeneration is difficult to judge because of ambiguity concerning which round bodies in the solution are protoplasts and which are vacuoles, etc. Counting all distinct round bodies as being protoplasts our efficiency of regeneration is approximately 5 percent.

Liposome preparation. Liposomes prepared using the ether injection method are large, unilamellar bodies. The liposomes prepared in our lab were sized by passing them through a 1.2 μm Millipore filter, with only 5 percent of the liposome suspension passing through. The amount of aqueous phase captured by the liposome was estimated by adding potassium chromate to the aqueous phase. After separating liposomes from free chromate by gel filtration chromatography, the amount of chromate in the liposomes was photometrically determined after correcting for the light scattering caused by the liposome suspension. We estimate that approximately 5 percent of the aqueous phase is captured in the liposomes.

Fusion experiments. The use of the met^- auxotroph, EP-6, as the recipient strain for cell-free infection assures that there will be no question of the origin of any TH strains that may result from cell-free infection experiments. The EP-113 is used as the source of dsRNA and membrane-bound dsRNA containing particles because of the high yield of both from this strain. We have attempted cell-free infection of protoplasts with free dsRNA, liposome captured dsRNA, and dsRNA containing membrane-bound particles. In attempting cell-free infection we combine equal volumes of protoplasts and the cell-free dsRNA preparation. After 2 hours of shaking at 100 rpm the suspensions are diluted and plated. Conidia from EP-6 were also plated onto the regeneration medium with and without salt as controls. Regenerated colonies are transferred to agar plates without NaCl and evaluated for colony color after 1 week. It was our assumption that dsRNA containing colonies would assume the white phenotype of EP-113 while those not containing dsRNA would retain the orange phenotype of EP-6 (Puhalla and Anagnostakis 1971; Van Alfen et al. 1978). The results of representative experiments are shown in Table 1. The relatively high percentage of regenerated colonies that were white suggested that we were successful in transmitting dsRNA. The controls however suggested that the white colonies resulted not from dsRNA transmission but rather from osmotic shock. Conversion experiments (Anagnostakis and Day 1979) using an orange strain of the same vegetative compatibility type, EP-34 (arg^-), indicated that the white color was not transmissible. Attempts to extract dsRNA from selected white regenerated colonies were also negative. Thus, the white colonies that resulted from our fusion experiments apparently did not contain dsRNA.

Discussion

The induction of high percentages of white colonies from orange ones by ultraviolet (UV) irradiation has previously been reported (Van Alfen et al. 1978). We can only assume that osmotic shock affects *E. parasitica* in an

Table 1. Percentages of white colonies after plating onto regeneration medium.[a/]

Propagules plated	Percentage of white colonies
Conidia plated onto regeneration medium without salt	0
Conidia	35
Protoplasts	4
Protoplasts + dsRNA	23
Protoplasts + dsRNA containing liposomes	24
Protoplasts + dsRNA containing membrane-bound particles	11

[a/] The composition of the regeneration medium is given in the text.

unknown way that results in a phenotype similar to that induced by TH and UV light. The lack of dsRNA in the sampled white colonies indicates that we have not successfully transmitted the dsRNA even though the expected phenotype occurred in high percentages. It is possible that we are losing the enzyme(s) necessary for replication or expression of the dsRNA.

This induction of white colonies by osmotic shock complicates our attempts of cell-free transfer of dsRNA. Our current plan is to test all white colonies for their ability to convert orange ones to white before determining whether they contain dsRNA. We also will increase the numbers of colonies tested. In past experiments we have transferred about 100 colonies per experiment for color development. In the different experiments 2 to 30 white colonies have been tested for ability to convert orange colonies to white with negative results. Obviously large numbers of whites will need to be tested in each experiment. A concerted effort to use naturally occurring membrane-bound particles may also prove useful if we are losing essential enzymes upon transmission.

The cause of variability in percentage of white colonies that are induced from experiment to experiment is not known. We know too little of the mechanism involved in this generation of high percentages of white colonies from virulent ones to speculate about such variability.

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STRATEGIES FOR ENHANCING DISSEMINATION OF HYPOVIRULENCE IN *ENDOTHIA PARASITICA*: STATE OF THE ART

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ABSTRACT.--*The chestnut blight epidemics of eastern United States and Italy were compared. In spite of substantial differences in their ecosystems (host species, forest community, rainfall distribution, major soil types), the epidemics caused by virulent (V) strains of Endothia parasitica proceeded apparently at similar rates, i.e. ca. 18 and 23 miles per year for eastern United States and Italy, respectively. This was facilitated by the abundant production of conidia, which are spread by a variety of agents, and wind-blown ascospores. Healing cankers attributed to hypovirulent (H) strains of the pathogen were observed in Italian chestnut forests 12 to 15 years after attack by V strains. Within 20 to 30 years, H strains succeeded in checking blight in Italy. The rate of spread of H strains in Italy, therefore, was about the same as that for V strains. In the absence of significant spread of H strains in the eastern United States during the past 80 years, it seems unlikely that such spread will occur naturally in the foreseeable future. It may, however, be possible to establish disseminating H strains by knowledgeable intervention. Perhaps the best hope for enhancing dissemination of H strains lies in an understanding of how hypovirulence spreads where it is spreading. Intensive studies in these areas (i.e. Italy, France, and perhaps most significantly, western Michigan) should seek to determine rates and patterns of spread, rates of increase of H strains, host density, incidence of vectors or other novel relationships affecting dissemination, contribution of asexual and sexual sporulation, and inoculum density of H strains. Slow rates of local spread need not preclude success as H strains can be established artificially in many places throughout the natural range of American chestnut. Chestnut blight continues unabated, with rare possible exceptions, in the eastern United States while the epidemic in southern Europe has subsided. This has provided hope that chestnut blight can be controlled eventually in the United States. An understanding of how hypovirulent (H) strains of Endothia parasitica were disseminated in Europe may bear heavily on the outcome of efforts to combat blight in North America. To address this question, it seems appropriate to first compare the epidemics of North America and Europe with particular emphasis on factors which could have influenced the dissemination of virulent (V) and H strains. This information could be instructive as we explore possible strategies to enhance and detect dissemination of H strains within the natural range of American chestnut.*

The Chestnut Blight Epidemics of Eastern United States and Italy

Most of the documented information about chestnut blight in Europe deals with the Italian epidemic. A comparison therefore will be made between the epidemics in eastern United States and Italy (Table 1). I confess an uneasiness about presenting the blight situation in Italy as I have not observed it personally. My interpretation of what has occurred is based solely upon a relatively sparse literature and personal communication. The source of uneasiness therefore lies in the danger of my misinterpretation of the printed word or my failure to have asked the right questions.

Table 1. Characteristics of chestnut - growing areas and of chestnut - blight epidemics in the eastern United States and Italy

	Eastern United States	Italy
HOST AND SITE		
Major host	*Castanea dentata* (Marsh.) Borkh.	*Castanea sativa* Mill.
Range	1200 x 500 = 600,000 miles^2	700 x 90 = 63,000 miles^2
Latitude	N 34° - 45°	N 38° - 46°
Major soil types	Gray-brown, red-yellow, podzolic soils	Calcimorphic, brown forest soils
Rainfall distribution	Throughout year	Summer drought
Prevailing wind direction	Southwest	Northwest
Forest community	Mixed hardwoods	Frequently pure, dense stands of selected cultivars
CHESTNUT BLIGHT EPIDEMIC		
First report of blight	1904	1938
Years to spread throughout range	ca. 40 years	ca. 30 years
Predominant direction of spread	Southwest	Variable
Rate of spread in predominant direction	900 miles ÷ 40 years = 23 miles/year	550 miles ÷ 30 years = 18 miles/year
Spread of hypovirulence through range	Negligible in 80 years	20 to 30 years

The chestnut blight epidemics of Italy and the eastern United States are separated by about 5,000 miles. It is not surprising, therefore, that these epidemics differ in major host species, associated plant and animal communities, and certain features of physical environment. Some of these differences will be discussed briefly with particular emphasis on how they may have effected dissemination of V and H strains of *E. parasitica*.

Dissemination of V Strains

While both the European and American chestnuts are highly susceptible to V strains, it is possible that the former is slightly less susceptible than the latter (Graves 1950). Most infection probably occurs during the growing season (Anderson 1913a; Double this proceedings). During moist conditions, asexual conidia are extruded from pycnidia and sexual ascospores are forcibly ejected through a film of water covering the ostioles of perithecia (Anderson and Babcock 1913). The production and dissemination of these spores, therefore, would be favored more by the moist summers of the eastern United States than by the dry summers characteristic of the Mediterranean climate of Italy (Watson 1968). Nonetheless, the average rate of spread in the predominant direction of spread was quite similar for the two epidemics. Blight was present throughout the range of American chestnut within about 40 years after it was first reported in New York City in 1904 (Beattie and Diller 1954). Spread of 900 miles to the Southwest therefore occurred at an average rate of about 23 miles per year. In Italy, blight spread throughout chestnut growing regions in about 30 years after it was first observed in the vicinity of Genoa in 1938 (Mittempergher 1978). Blight in Italy therefore spread 550 miles to the Southeast at an average rate of about 18 miles per year.

The rate of disease increase in American chestnut was extremely rapid in some areas. For example, detailed observations were made in a small area within the advancing edge of infection in the vicinity of Bluemont, Virginia, during 1913 to 1914 (Rogers and Gravatt 1915). This community is about 20 miles east of Winchester and 250 miles southwest of New York City. Of the 140 chestnut trees in this area of about 1,300 m^2, 50 (29 percent)were infected in 1913 and 83 (59 percent)were infected in 1914. From this data a rate of disease increase (r) of 1.26 can be calculated (Vanderplank 1963). At this rate, disease would increase from 1 percent to 90 percent in 5 to 6 years. This is in general agreement with the rates of disease increase observed in study plots in Maryland and Virginia (Gravatt and Gill 1930). While the number of infected trees doubled in 1 year in the Bluemont study, it is interesting to note that the total area and number of cankers increased by factors of about 5 and 3, respectively, during the same time. This information provides an opportunity to relate canker area, which was ca. 19,300 cm^2 at the 1913 observation, with rate of disease increase. Merrill (1967) reported r values of 1.42 and 0.83 for the chestnut blight epidemics in Pennsylvania and Connecticut, respectively. I have not seen data that would permit the calculation of r values for the Italian epidemic.

Ascospores are wind disseminated after their forcible ejection from perithecia. It has been suggested that these spores played a major role in the spread of V strains (Anderson 1913b; Heald et al. 1915). It is curious, however, that spread in Italy appeared to be multidirectional (Baldacci and Orsenigo 1952) with a prevailing northwest wind, and the most rapid spread in the eastern United States was to the Southwest (Metcalf and Collins 1911; Gravatt and

Marshall 1926) into a prevailing southwest wind. It could be argued that spread was in the direction of the greatest concentration of susceptible hosts. But, the early spread of the wind-disseminated gypsy moth was to the North and Northeast, i.e. with the prevailing wind, after its introduction into Massachusetts in 1869 (Campbell 1979), even though the colder northern temperatures are sometimes lethal to eggs (Summers 1922) and the greater concentration of preferred hosts is to the South. While the significance of this is unclear, it may be a reflection of the more rapid growth of the pathogen and concomitant production of inoculum in the warmer South as suggested by Stevens (1917). Lack of correlation between prevailing wind direction and direction of spread also suggests that agents of dissemination in addition to wind-blown ascospores could have played a significant role in disease spread. Indeed, some additional agents have been implicated. Those mentioned most prominantly were insects, birds, and the movement of infected host material by man.

The major differences in geographic area, soil type (Watson 1968), and plant community dictate also major differences in the insect fauna associated with the epidemics of eastern United States and Italy. Representatives of major insect taxa associated with one epidemic, however, would also be expected to be associated with the other. Insects are the vectors of many plant pathogens (Harris and Maramorsch 1980; Leach 1940), and they received early attention as possible vectors of chestnut blight fungus. Although viable spores were collected from a variety of insects including ants and beetles (Anagnostakis this proceedings; Anderson and Babcock 1913; Studhalter and Ruggles 1915) and mites (Wendt et al. this proceedings), I am unaware of any work that clearly satisfies the rules of proof for insect transmission (Leach 1940). Consequently, opinion was mixed as Studhalter and Ruggles (1915) concluded that insects were important vectors whereas Craighead (1916) concluded otherwise because the insects that frequent blight cankers rarely visit healthy trees. Craighead suggested that the significance of insects in disease dissemination was in their providing suitable wounds for infection. While this may be true, it has not been established experimentally. Of the many types of wounds inoculated by Anderson and Babcock (1913), insect holes were among the very few that did not become infected. This could reflect an effect of wound condition or age. We need to know more about what constitutes an infectable wound, particularly as it may relate to the dissemination of H strains.

Conidia of *E. parasitica* were recovered from the bodies of six species of birds in Pennsylvania during the winter and spring of 1913 (Heald and Studhalter 1914). Most of these birds which included the brown creeper, *Certhia familaris;* downy woodpecker, *Dendrocopus pebescens;* golden-crowned kinglet, *Regulus satrapa satrapa;* Junco, *Junco hyemalis;* white-breasted nuthatch, *Sitta carolinensis;* and sapsucker, *Sphyrapicus varius;* were killed either during or shortly after visiting chestnut blight cankers. Birds shot within 2 to 4 days of significant rainfall carried the highest number of conidia (up to 757,000). The cedar waxwing, *Bombycilla cedrorum,* recently was added to the list of birds that has carried inoculum (Scharf and DePalma 1981). Circumstantial evidence therefore suggests that vectoring could have occurred by highly active mobile birds that visit both cankered and healthy trees. The sapsucker seems to be a particularly viable candidate in that it is migratory and it feeds on insects as well as sap obtained from wounds it inflicts into the xylem of healthy trees (Bent 1939). Some of the genera of birds that carried spores in the eastern United States occur also in Italy, e.g. creepers,

woodpeckers, kinglets, nuthatches, and waxwings (Bruun and Singer 1970). Small mammals have been implicated as possible vectors for local spread (Scharf and DePalma 1981).

Movement of infected host material by man probably provided the inoculum for some of the infection centers that occurred well in advance of the main epidemic (Anderson and Babcock 1913; Gravatt and Marshall 1926). There is little doubt that such movement enabled V strains to gain initial entry into the eastern United States and Italy. Differences in the culture of chestnut in the two areas suggest that movement of infected host material may have played a greater role in dissemination in Italy. The American chestnut occurred usually in natural stands of mixed hardwoods whereas chestnut frequently is grown in pure dense stands of selected cultivars in Italy (Mittempergher 1978). The intensive culture of chestnut in Italy, including movement of specific cultivars throughout the country, therefore offered considerable opportunity for the movement of infected material.

The rapid rate of spread of V strains in both the eastern United States and Italy demonstrates that efficient means for dissemination were not limiting.

Dissemination of H Strains

Healing cankers were first observed near Genoa, Italy, about 12 years after the first report of blight in the same area. Healing cankers soon were observed in other areas of older infection, i.e. about 15 years after initial blight (Biraghi 1953; Mittempergher 1978) and within 20 to 30 years H strains "succeeded in checking the disease in Italy" (Grente and Berthelay-Sauret 1978). This indicates that the rates of dissemination of V and H strains were similar in Italy. The rate of increase of H strains also was substantial. The percentage of H strains isolated from cankers in the Piedmont increased from about 25 percent in 1972 to about 75 percent in 1977 (Grente and Berthelay-Sauret 1978). From this an r value of 0.44 can be calculated. If this rate of increase were sustained, H strains would increase from 1 percent to 90 percent in 15 to 16 years. The rate of increase of H strains also was considerable following artificial inoculation of cankers with H strains in France. An r value in excess of 0.68 can be calculated if we assume from data provided by Grente and Berthelay-Sauret (1978) that a hectare was "completely healed" 10 years after 10 of its 100 cankers were treated with H strains. Radial spread of H strains around inoculation sites proceeded at 1 to 2 m per year. While this figure is not impressive, particularly to some (Kuhlman 1981), it may not be as bad as it seems because the area of spread is a function of the square of the radius. The area of spread therefore quadruples with each doubling of time after successful inoculation. For example, a radial spread of 2 m per year translates into an area of spread of 314 m^2, 1,257 m^2, and 5,027 m^2 after 5, 10, and 20 years, respectively. It might be mentioned that the annosus root-rot epidemic also spreads locally at a rate of about 1 m per year (Berry 1968). Few would argue that this disease spreads too slowly to be of consequence.

In an evaluation of possible causes for the gross difference in spread of H strains in Italy and the eastern United States, differences in major host species should not be ignored. It is conceivable that host responses to H strains by *C. sativa* are more favorable than those by *C. dentata* for the development of the type cankers which are most conducive for dissemination

(e.g. persistent, nonlethal) (Elliston 1981). The recent observations of Weidlich et al. (this proceedings) and Brewer (this proceedings), however, suggest that American chestnut is capable of supporting this type of H canker in Michigan.

It seems unlikely that windblown ascospores played a role in the dissemination of H strains in Italy. Few perithecia are produced by H strains (Elliston 1978; Turchetti 1978) and the dsRNA associated with hypovirulence has not been shown to be transmitted through the sexual stage (Anagnostakis personal communication). The role of conidia is less clear. Fewer pycnidia are produced by H strains than by V strains (Elliston 1978) but dsRNA sometimes is transmitted to conidia by the former (Anagnostakis; Elliston personal communication). The number of conidia containing dsRNA would dictate the potential of these spores for dissemination of H strains.

As with V strains, other agents that could have played a role in the dissemination of H strains include birds, small mammals, insects, and the movement of infected host material by man.

Even though some of the genera of birds that carried inoculum of V strains in the eastern United States occur also in Italy, as mentioned above, it seems unlikely that they played a significant role in the recent dissemination of H strains. During a 3-week tour in 1978, Elliston (personal communication) did not see or hear birds or small mammals in the chestnut forests he visited in Italy. Evidence of woodpecker feeding at cankers also was not observed. Turchetti (personal communication) indicated to Elliston that the bird and squirrel populations of Italian forests have been hunted almost to extinction.

Prospects for insect dissemination of H strains seem better although information on insects associated with blight cankers in Italy is lacking. The serious reservations of Craighead (1916) with regard to dissemination of V strains are not as applicable to H strains. As pointed out earlier (Day 1978), insects which frequent blight cankers could facilitate movement of H inoculum to V cankers. The mention by Mittempergher (1978) that the pure chestnut stands are excellent grazing grounds suggests that they may be used extensively for this purpose in Italy. This could effect among other things, the number and species of insect residents. Ants were mentioned by Grente (Day 1978) as possible vectors of H strains in France. Studies on possible insect vectoring of H strains, including the use of surrogate fungal attractants, were reviewed earlier (Russin et al. this proceedings).

The extensive movement of host material by man in Italy could have played a more important role in the long-distance spread of H strains than V strains due to the limited sporulation of the former.

Prospects for the Dissemination of H Strains in the eastern United States

Hypovirulent strains spread through the Italian chestnut forest in 20 to 30 years (Grente and Berthelay-Sauret 1978) or about as quickly as V strains. In the absence of significant spread of H strains in the eastern United States during the past 80 years, it seems unlikely that such spread will occur naturally in the foreseeable future. It may, however, be possible to establish disseminating H strains by knowledgeable intervention.

Perhaps our best hope for enhancing dissemination of H strains lies in an understanding of how hypovirulence spreads where it is spreading (e.g. Italy, France, and perhaps most significantly, western Michigan). The striking similarity between what has happened in Italy and what is happening in western Michigan (Mittempergher 1978; Weidlich this proceedings; Brewer this proceedings) is exciting. Of particular interest is the characteristic lag of about 15 years between V-strain epidemic and the observation of healing cankers. One cannot help but wonder about the source(s) of hypovirulence in these widely separated areas. Intensive studies in these areas should seek to determine the following: rates and patterns of spread; rates of increase of H strains; host density; incidence of vector or other novel relationships affecting dissemination; contribution of asexual and sexual sporulation to dissemination; inoculum density of H strains. With regard to the last point, previous studies by Rogers and Gravatt (1915) and Hebard et al. (1981) have provided some information on the relationship between canker area and rates of disease increase for V strains. Information on how much canker area is required for appreciable spread of H strains is lacking but it probably would be greater than the 500 $cm^2/400\ m^2$ plot reported for V strains (Hebard et al. 1981) because of the reduced sporulation on H cankers.

In concomitant studies in other areas, natural or derived H strains could be evaluated for their capacity to spread. Strains with nuclear (auxotrophic, abnormally pigmented) and/or cytoplasmic (specific component(s) of dsRNA) markers could be utilized for monitoring dissemination. Such studies should take into consideration the possible limitations of vegetative incompatibility, inoculum density, wound susceptibility, and host density.

There is a need for additional information on the basic biology of hypovirulence. Such information could assist in the development of H strains that are most efficient in achieving their assigned task. It is conceivable, for example that certain fragments of genomes of dsRNA permit greater sporulation and carry over of transmissible hypoviurlence in spores.

Slow rates of local spread need not preclude success as H strains can be established artificially in many places throughout the natural range of American chestnut.

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HOST-PARASITE INTERACTIONS OF *ENDOTHIA PARASITICA* ON CHESTNUT SPECIES: STATE OF THE ART

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ABSTRACT.--*Cankers incited by Endothia parasitica on American, European, Japanese and Chinese chestnut trees have been described for various parts of the world and may be classified into four general types: A) sunken, B) irregularly swollen, C) callused and swollen ((i) with and (ii) without necrosis of the callus bark tissue), and D) superficial and swollen. Intermediate forms among A to D have been described and more than one canker type may be observed on the same tree. Canker types Cii or D have been associated commonly with virulent E. parasitica on Chinese and Japanese chestnut trees and hypovirulent E. parasitica on American and European chestnut trees, whereas canker types A, B or Ci are commonly associated with virulent E. parasitica on American, European and sometimes on Japanese and Chinese chestnut trees. Environmental factors (cold injury, frost injury, low soil fertility, shading or competition, and low soil water potential) that stress the host appear to play central roles in increasing the development of virulent E. parasitica on the normally blight-resistant Oriental chestnut species. The extent and nature of wound periderm formation appear to be important factors limiting E. parasitica infections in canker types Cii and D, but wound periderm appears to be ineffective, in most instances, in canker types A, B and Ci, due to a rapid growth rate of mycelial fans of the pathogen in bark tissues. In American and European chestnut trees, wound periderm may not be formed or is often not fully formed when the virulent pathogen invades this and neighboring tissues. Tannins (especially hamamelitannin in Castanea dentata and C. sativa), carbohydrates and amino acids in bark tissues appear to be key energy, carbon and nitrogen sources supporting rapid E. parasitica mycelial fan growth. The mechanical forces exerted by the mycelial fan, oxalic acid toxicity, acidification of host tissues (partly through the production of oxalic acid), and hydrolytic enzymes may play important roles in pathogenesis by E. parasitica.*

The pioneering histopathological and histochemical research of Keefer (1914) and Bramble (1936) laid the foundation for our understanding of the host-parasite interactions of *Endothia parasitica* on American chestnut *Castanea*

dentata. Subsequent work by Bazzigher (1955; 1957) of a histopathological and physiological nature provided new insights and permitted a comparison of the behavior of *E. parasitica* on European *C. sativa* and American chestnut trees. Today, hypovirulence in *E. parasitica* and blight resistance in Chinese chestnut *C. mollissima* offer contrasting systems to determine the key factors limiting canker development on chestnut, in hope that this understanding will enable us to better control virulent forms of the pathogen.

Cankers incited by *E. parasitica* on *C. dentata*, *C. sativa*, Japanese chestnut, *C. crenata* and *C. mollissima* have been described for various parts of the world (Biraghi 1953; Bramble 1936; Bonifacio and Turchetti 1973; Heald 1913; Headland et al. 1976; Jones et al. 1980; Mittempergher 1978; Uchida 1977) and may be classified into four general types:

A) Sunken. Cankers rapidly expanding with a sunken and commonly split surface. Infection extends to the vascular cambium, and death is typically observed. Fructifications are typically abundant, particularly on smooth bark. Often the canker margin is slightly swollen or raised on American, European and Japanese chestnuts.

B) Irregularly swollen. Cankers with irregularly swollen and sunken areas due to progression and cessation of lesion and host tissue growth. Splitting and sloughing of the bark may occur. Infection often extends to the vascular cambium. Fructifications are abundant to sparse on American, European, Japanese and Chinese chestnuts.

C) Callused and swollen. Cankers characterized by early progressive infection of bark tissues to the vascular cambium with one or more prominant callus (differentiated xylem and phloem) ridges ringing the deep infection, and with (i) or without (ii) invasion or necrosis of the callus tissues by *E. parasitica*. Exposed wood may be present at the canker center and on the older callus ridges that give a swollen appearance to the stem. Infected tissues may slough completely away in some instances. Fructifications are sparse to moderately frequent on American, European, Japanese and Chinese chestnuts.

D) Superficial and swollen. Cankers with superficial infection of small or large areas of the bark that typically give a swollen appearance to the bole or branch. Little or no infection extends to the vascular cambium in most instances. There may be extensive sloughing of infected tissues. Fructifications are sparse to nonexistent. Large canker areas may be observed on American and European chestnut trees and small areas may be observed on Chinese and Japanese chestnut trees.

Intermediate forms among A to D have been described and more than one canker type may be observed on the same tree.

Canker types Cii or D have been associated with virulent *E. parasitica* on Chinese and Japanese chestnut trees and hypovirulent *E. parasitica* on American and European chestnut trees, whereas canker types A, B or Ci are commonly associated with virulent *E. parasitica* on American, European and sometimes on Japanese and Chinese chestnuts.

In a study conducted over 13 provinces of Italy, Bonifacio and Turchetti (1973) isolated normal (virulent) strains of *E. parasitica* from 9 of 13

restricted (=healed) or partially restricted cankers on *C. sativa*. Nine of 13 restricted or partially restricted cankers yielded morphologically atypical strains that were hypovirulent. From four of 13 cankers of these two types only atypical strains were isolated. All of nine nonrestricted cankers yielded normal strains and seven of these yielded only normal strains. All of four restricted cankers yielded at least one atypical strain type. Thus, restricted cankers were generally, although not exclusively, associated with the presence of atypical strains.

Mittempergher (1978) distinguished two types of restricted cankers in Italy, which correspond to the superficial swollen (type D) and callused swollen types (type Cii) above. Bark swelling and low pycnidium production were associated with both. The superficial cankers covered large areas of the stem. The bark was slightly cracked and appeared rough and dark in color. Cork or wound periderm appeared to effectively restrict *E. parasitica* colonization of tissues toward the vascular cambium and contributed to the sloughing of infected tissues. The second canker type exhibited *E. parasitica* infections to the vascular cambium, and mycelial fans were evident on the xylem. Wound responses were observed in swollen areas surrounding the deep infection and these restricted further canker development. Presumably, the latter was due to the infection of bark tissues by a hypovirulent strains(s) subsequent to the infection of the vascular cambium by a virulent strain(s).

Restricted or, more commonly, partially restricted cankers have been observed by us on large, surviving and stump-sprout American chestnut trees in the Appalachian region of the United States. Some of these cankers correspond to the healthy, callused-swollen (type Cii) or superficial-swollen type (type D), but most correspond to the necrotic, callused-swollen type (type Ci) according to our observations. Sometimes an individual tree will have all three canker types. The presence of the latter type may be associated with death of that portion of the stem or tree if invasion of the callus and inner bark is extensive. Crown ratings (percent of branches not killed) for surviving American chestnut trees over 25 cm d.b.h. are commonly over 50 percent. For one such tree in northern Virginia we found approximately one-third of 100 bark-tissue isolates of *E. parasitica* were hypovirulent in inoculation trials on American chestnut stump sprouts. Superficial swollen cankers were common on this tree, as indicated by examination of corkborer bark samples from the extensively cankered stems. Lower percentages (10 to 30 percent) of hypovirulent *E. parasitica* were found in 12 other surviving American chestnut trees and associated smaller American chestnut trees in northern Virginia, based on trials with 10 bark-tissue *E. parasitica* isolates per tree. In an earlier study (Griffin et al. 1978), using one isolate per tree, and in a more recent study, using 30 isolates from a single West Virginia tree, we found little association of hypovirulent *E. parasitica* with partially restricted cankers on large, surviving American chestnut trees. Jaynes (1981) surveyed 20 large (mostly > 30 cm d.b.h.), surviving American chestnut trees and found that many of the selected abnormal isolates from these trees were hypovirulent. Further research is required on determining the levels of *E. parasitica* hypovirulence and blight resistance in such trees before we can make a reliable assessment on how much hypovirulence contributes to the survival of large, surviving American chestnut trees. Through inoculation trials with virulent *E. parasitica*, we have found that several surviving trees have various levels of blight resistance that may be a factor in survival. Trees with superficial swollen cankers or callused swollen cankers

are the most likely candidates for this research. Small superficial cankers are found on blight-resistant Chinese chestnut, from our observations, and Uchida (1977) has described cankers on normally blight-resistant Japanese chestnut that may be of this type. He reports that the necrotic tissue is often sloughed off from these cankers.

Wound periderm formation is typically associated with successful attempts by chestnut species to limit infection by both virulent and hypovirulent *E. parasitica*, and appears to be a principal tissue, along with phloem parenchyma, that is associated with swollen, superficial cankers on chestnut boles or branches. As indicated above, the bark swelling feature is often associated in the same canker with sunken or killed bark tissues or with exposed wood tissues, even in blight-resistant Chinese chestnut. Histopathological studies by Bramble (1936) on American chestnut and by Bazzigher (1957) on European chestnut, infected with virulent *E. parasitica*, indicate wound periderm, composed of about eight cork-cell layers in the phellem, is effective in blocking colonization by *E. parasitica*. However, Bramble (1936) noted that complete infection of American chestnut bark by virulent *E. parasitica* was associated with the absence of wound periderm formation. Both Bramble (1936) and Bazzigher (1957) observed that the virulent fungus could advance in bark tissues containing wound periderm in areas where wound periderm had not yet formed. Similarly, Grente and Berthelay-Sauret (1978) reported that small islands of cork cells, not forming a continuous barrier, were formed in European chestnut bark tissues infected with virulent strains. With hypovirulent *E. parasitica* strains, a complete cork barrier was observed. These observations raise the question as to whether the rate of wound periderm formation, the completeness of wound periderm formation or the thickness of wound periderm formation are important in restricting canker development. The lignified zone, or wound periderm-induction barrier (WPIB), does not limit infection by the pathogen (Bramble 1936). To examine the above aspects, Hebard et al. (1979) inoculated highly blight-resistant Chinese chestnut ('Nanking'), a moderately blight-resistant, surviving American chestnut in Virginia and blight-susceptible American chestnut stump sprouts with a killing strain of *E. parasitica* and with hypovirulent (slightly and weakly pathogenic) strains of the fungus. They found few differences in the initiation time for the formation of the WPIB or wound periderm, among the different trees and for inoculated and noninoculated wounds, in the secondary phloem over the first 10 to 20 days. Thereafter, maturation and completeness of wound periderm formation lagged in the secondary phloem and cortex of blight-susceptible stump sprouts inoculated with the virulent strain, and the formation of wound periderm was less uniform for this combination. In many instances no wound periderm was formed in the blight-susceptible trees. For all chestnut types, wound periderm formation occurred first in the secondary phloem near the vascular cambium and developed later in the cortex or outer bark tissues. For moderately blight-resistant American chestnut inoculated with virulent *E. parasitica*, wound periderm in the secondary phloem and cortex blocked further infection by the fungus, except in the cortex where there was a small or large discontinuity in the three- or four-cell thick wound periderm or where a mycelial fan had apparently pushed laterally through a two-cell thick wound periderm. In Chinese chestnut inoculated with the virulent strain, the fungus was restricted, although not always, by wound periderm that formed as rapidly, as completely and as thickly (about eight cells) in the inoculated wounds as in the noninoculated wounds. With a few exceptions, no gaps or thin wound periderm areas were

noted in the secondary phloem and cortical wound periderm. Similar results were found for the slightly pathogenic Italian hypovirulent strain EP-66, on highly blight-resistant Chinese chestnut and moderately blight-resistant American chestnut, but this strain on blight-susceptible American chestnut was associated with the formation of significantly fewer cell layers in the wound periderm, compared to the noninoculated control or other low-disease, pathogen-host combinations.

Research by Hebard et al. (1979) has suggested that the dominant factor in host-parasite interactions of a progressive, lethal canker is the rapid formation and growth of the mycelial fan. The wedge-shape mycelial fan of the virulent pathogen on blight-susceptible American chestnut appears to exert a physical pressure that the lignified zone or tissues other than a many-cell-layered wound periderm cannot withstand (Bramble 1936; Hebard et al. 1979; Keefer 1914). Splitting of bark tissue in front of the fan was commonly observed by Hebard, Griffin and Elkins (unpublished data). In addition, cell death occurred at least 350 μm in advance of the fan in the absence of a lignified zone, as indicated by neutral red straining. If fans were close to the newly forming lignified zone or WPIB, this death may have prevented wound periderm formation. Histopathological observations made by Hebard et al. suggest this may occur. Grente and Berthelay-Sauret (1978) have suggested the *E. parasitica* toxin, diaporthin, may prevent wound periderm formation, but McCarroll (1978) found no evidence that diaporthin was present in cankered tissues on American chestnut. McCarroll (1978) proposed that oxalic acid production and acidification of host tissues by *E. parasitica* played important roles in pathogenesis in front of the mycelial fan. It is possible that oxalic acid and other acidic agents may interfere with wound periderm formation after the mycelial fan breaks through the lignified zone. McCarroll (1978) reported there was a drastic decline in the pH of bark tissues associated with recent infection. He found that the pH values of bark tissues in the uninfected areas, the gelatinous zone ahead or the fan (probably the brown or yellow-brown zone described by other workers), and advancing edge of mycelium were 5.5, 4.7 and 2.8, respectively. Oxalic acid concentration increased with infection and was 3.8 to 1.1 mg/g bark in uninfected areas and 9.3 mg/g bark in area of the gelatinous zone and leading 0.5 cm of mycelium. In bioassays, oxalic acid caused a browning of cells near the vascular cambium.

Hebard, Griffin and Elkins (unpublished data) observed that virulent *E. parasitica* produced multiple, rapidly growing mycelial fans in susceptible American chestnut stump sprouts and one was frequently located at the vascular cambium. In contrast, slower-growing fans were formed by two slightly pathogenic hypovirulent isolates (American W-2 and Italian EP-66), and none were observed for two weakly pathogenic hypovirulent isolates (EP-14 and EP-3) in blight-susceptible American chestnut. In moderately blight-resistant American chestnut and highly blight-resistant Chinese chestnut, typically only one fan was formed by virulent *E. parasitica*, and this was located in the outer bark tissues where, as mentioned, wound periderm formation occurred later. The combination of these two factors (one fan formation and late wound periderm formation in the cortex) appear to be the key elements to explain the superficial nature of many cankers caused by virulent *E. parasitica* on blight-resistant chestnut trees and by hypovirulent *E. parasitica* on blight-susceptible chestnut trees. In the latter instance, the slower rate of fan formation and growth of the hypovirulent isolate, compared to the

virulent isolate, may permit more complete formation of wound periderm in the secondary phloem next to the vascular cambium. In the cortex, where wound periderm formed later, the pathogen is able to advance superficially. No mycelial fans were formed by hypovirulent *E. parasitica* on moderately or highly blight-resistant chestnut trees in the Hebard et al. study.

The rapid growth and multiple formation of mycelial fans by virulent *E. parasitica* in blight-susceptible American chestnut may be dependent in part upon the high availability of utilizable tannins in this species. Other carbon- and nitrogen-containing energy sources, such as carbohydrates and amino acids, are likely to be important as well. Although tannins may be toxic at high concentration (Nienstaedt 1953; Uchida 1977), Cook and Wilson (1916); Bazzigher (1955) and Uchida (1977) demonstrated that *E. parasitica* utilized the tannins of American, European and Japanese chestnut trees, respectively. Significantly, Bazzigher (1955) found that tannins were removed from European chestnut bark during canker development. Elkins et al. (1978) found that utilization of tannins from aqueous bark extracts of American, European and Chinese chestnut trees was greater for the two blight-susceptible species than for the blight-resistant species. Similarly, the mycelial growth (dry weight) of *E. parasitica*, on which fan formation is dependent, produced on extracts of bark was greater for the two blight-susceptible species than for the blight-resistant species. A pH drop following growth, possibly due to oxalic acid production, was observed for all extracts. Elkins et al. (1979) found that bark tissues of blight-susceptible American and European chestnut trees contained high amounts of hamamelitannin whereas none was found in blight-resistant Chinese and Japanese chestnut trees. Hamamelitannin was rapidly utilized for growth by *E. parasitica* in axenic culture.

Bramble (1936) made observations that suggest the browning reaction at the margin of the mycelial fan may be due to tannin oxidation or to polymerization of tannins. At the margin of the fan, the color intensity of the host cells increased initially, upon treatment with $FeCl_3$, which was followed by no color when the cell contents had turned yellow-brown. Both he and Hebard, Griffin and Elkins (unpublished data) also observed a greater $FeCl_3$ reaction in the area of wound periderm formation, suggesting mobilization of phenolics by the host in that area. Biochemical defense mechanisms in blight-resistent chestnuts may be important also in limiting fan formation in blight-resistant chestnuts. McCarroll (1978) demonstrated that Chinese chestnut tissues displayed a greater sensitivity to oxalic acid and acidification than American chestnut and suggested that hypersensitivity (as indicated by browning) of Chinese chestnut to these agents may be a factor in blight resistance. He suggested that pathogen enzyme denaturation and pectinase inhibition by the host were also important factors.

Environment appears to be a dominant factor in host-parasite interactions of virulent *E. parasitica* on blight-resistant Chinese and Japanese chestnut trees, but little is known about the effect of environment on the interaction of hypovirulent *E. parasitica* and blight-susceptible American and European chestnut trees. Uchida (1977) has shown that low soil fertility (especially nitrogen), low soil water potential, shading or competition, frost injury or cold injury were important in increasing the susceptibility of Japanese chestnut to *E. parasitica*. He found also that seedlings were more susceptible than older trees. Observations of Berry (1951) and surveys of Jones et al.

(1980) in the United States suggested that frost injury or cold injury or both were associated with increased susceptibility of Chinese chestnut trees to *E. parasitica*. Thus, environmental factors that stress the host appear to be important factors in affecting host-parasite interactions. These stress factors may affect the ability of the host to rapidly form a continuous and fully developed wound periderm or to effect biochemical defense. In both large, surviving American chestnut trees and Chinese chestnut trees, we have observed, following canker dissection, instances where the advance of the pathogen in the bark was restricted, except where small radially advancing "leads" of necrotic tissue had progressed deeply below the generally contained infection. Similarly, in these trees we often observed a local area of infection at the vascular cambium, as evidenced by small areas of exposed wood or necrosis of the vascular cambium at the canker center. Apparently, this occurred under conditions stressful to the host. Subsequently, the host formed callus tissues, with differentiated xylem, phloem and wound periderm that excluded the pathogen, when conditions became more favorable to the host. As stated previously, often a series of four or five exposed xylem ridges may be observed for cankers on surviving American chestnut trees or on Chinese chestnut trees, suggesting a periodicity of environmental conditions unfavorable and favorable to defense against *E. parasitica*. Using regression analysis, Uchida (1977) found the extent of callus development by Japanese chestnut (probably an indicator of tree vigor) was inversely related to canker development. In American or European chestnut infected with hypovirulent strains, differences in the ability of the host to limit infection of *E. parasitica* in an individual canker also may be a reflection of the relative proportion of virulent or hypovirulent thallus in the canker. Areas in the canker where necrotic "leads" extend beyond the generally limited canker may be areas where virulent strains are present.

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MOLECULAR ASPECTS OF HYPOVIRULENCE: STATE OF THE ART

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ABSTRACT.--*Transmissible hypovirulence has been consistently associated with the presence of one or more components of high molecular weight dsRNA. Unfortunately the direct proof, by cell-free transmission, that the dsRNA is the hypovirulent factor is still lacking. Evidence would suggest that this dsRNA may be a biologically unique entity because it lacks a protein capsid. It is also unique because the numbers of dsRNA components present may be variable. Nothing is known of the relationship of the dsRNA of one strain to that of another. There are both transmissible and non-transmissible forms of hypovirulence of E. parasitica. While there are undoubtedly many forms of non-transmissible hypovirulence, there may be biochemical or genetic simularities between transmissible and some types of non-transmissible hypovirulence. A better knowledge of the genetics and control of virulence of E. parasitica is needed to understand how dsRNA or other factors may reduce the virulence of this pathogen. Fortunately, the fact that virulence of E. parasitica can be perturbed in a number of ways can be utilized to better understand the genetics of virulence and hypovirulence.*

Hypovirulence of *Endothia parasitica* has caught the imagination of people because it offers hope for the restoration of the American chestnut. As a result, most of the research concerning hypovirulence has justifiably emphasized field applications of this biological control phenomenon. The molecular aspects of hypovirulence, however, are equally exciting. The control of virulence expression in a pathogen by cytoplasmic genes has been an area of rapid and exciting discovery. For instance, it has been found that plant tumor induction caused by *Agrobacterium tumefaciens* (Merlo 1978) and *Pseudomonas savastanoii* (Comai and Kosuge 1980) are controlled by plasmid genes. Until the discovery of hypovirulence of *E. parasitica*, however, all of the known transmissible virulence control systems acted to increase the virulence of the pathogen. Hypovirulence of *E. parasitica* is unique because the cytoplasmically transmissible genes reduce pathogen virulence. Study of this system may provide researchers with clues concerning the genetic control of virulence and perhaps point the way to creation of similar hypovirulence control systems in other pathogens.

These research goals are naturally speculative and their realization years ahead. For the immediate goal of improving field spread of hypovirulence, knowledge of the molecular aspects of hypovirulence of *E. parasitica* is imperative. Our current knowledge of the nature of these cytoplasmically

transmissible hypovirulence control genes is very limited. To understand how they may spread and the limitations to their spread we must first know something of their biological nature. There is good correlative evidence that the hypovirulence factor genome is encoded on double-stranded (dsRNA). Direct evidence for this is needed however. We must also determine how the dsRNA is packaged within the fungal cell since this may be a critical factor in determining how spread occurs, or is limited. Finally, there has been much speculation concerning the origin and possibility that we are dealing with more than one hypovirulence factor. These questions cannot be answered without knowing much more about the genome and packaging of the hypovirulence factor.

In this short discussion of the molecular aspects of hypovirulence we have identified four different questions that we feel should be addressed as an initial step in understanding this phenomenon. The first three are very basic questions concerning the nature of the hypovirulence factor: 1) Is dsRNA the genome of transmissible hypovirulence? 2) Is the dsRNA associated with hypovirulence the genome of a virus? 3) What is the nature of the dsRNA associated with hypovirulence? The fourth question is: How does the dsRNA regulate the virulence of *E. parasitica*? This last question is not likely to yield answers as readily, in response to a researcher's efforts, as the first three. It is an important one, however, since it may hold the key to the success of expanding hypovirulence to other systems. We have organized our discussion to address what is currently known about each of these questions, and to make suggestions concerning what we need to know.

Is dsRNA the Genome of Transmissible Hypovirulence?

It is universally accepted among those working with hypovirulence of *E. parasitica* that the dsRNA extracted from hypovirulent strains is responsible for the control of hypovirulence expression. Unfortunately, the evidence for this is all correlative, and thus the conclusion so universally accepted may be wrong. There is a need for direct evidence of the role of dsRNA in hypovirulence, such as the cell-free transfer of dsRNA into virulent cells with the resultant transfer of the hypovirulence phenotype. One of the reasons for the confidence in the correlative evidence is that it is good. It has been shown that 1) as dsRNA is carried with the cytoplasm of a transmissible hypovirulent (TH) strain into a virulent (V) one during hyphal anastomosis, the TH phenotype follows the dsRNA (Anagnostakis and Day 1979), 2) as dsRNA is lost from a TH strain, the TH phenotype is also lost (Van Alfen and Gillies unpublished data), and 3) specific TH phenotypes associated with specific dsRNA segments follow those segments upon transmission (Elliston 1981). At least one of the isolates (EP-49) that contains dsRNA has been judged to have comparable virulence to some isolates that do not contain dsRNA (Elliston 1978). This would seem to negate the correlative evidence between hypovirulence and dsRNA. However, some of the conidia obtained from this isolate are typically hypovirulent and contain dsRNA (Van Alfen et al. 1978a). Thus, the presence of dsRNA within the thallus of this isolate can be correlated with the potential of producing typical TH progeny.

One of the problems with correlative evidence is that subjective judgments must be made. Since virulence in *E. parasitica* is a continuum (Elliston 1978),

there is a point where a line must be drawn to distinguish virulent from hypovirulent isolates. One is naturally inclined to draw that line between TH and V at the point where dsRNA is no longer found. Direct evidence would reduce the possibility that the prejudice of the investigator is influencing the conclusion.

Cell-free infection of V strains by the dsRNA if simple, would have been accomplished by now. It has been attempted in more than one laboratory. Even the simpler problem of cell-free infection of fungi by intact mycoviruses has been successfully reported only a few times (Ghabrial 1980). The advantage mycoviruses would have over naked dsRNA for cell-free infection is that mycoviruses are known to carry the RNA polymerases necessary for their replication (Buck 1980). All dsRNA mycoviruses or dsRNA alone require such enzymes for replication. Cell-free infection with phenol extracted dsRNA may thus be an impossible task since any associated RNA polymerases would be removed by the phenol. Initial replication of the dsRNA would be dependent upon enzymes present in the fungus. Knowledge of how the dsRNA is packaged and whether RNA polymerases are present within the package may help facilitate cell-free transfer to fungal protoplasts.

In our laboratory we are attempting cell-free infection by packaging the dsRNA within artificial membrane liposomes for transport of the dsRNA into the protoplast of virulent strains (Van Alfen and Hansen 1982). We are also attempting cell-free infection of protoplasts using the membrane-bound vesicles containing dsRNA that can be extracted from TH strains (Dodds 1980a). These dsRNA containing vesicles are fused with protoplasts using the same methods as employed for artificial membrane liposomes. Hopefully, one of these methods of introducing the cell-free preparation of dsRNA into virulent strains will be successful.

Is dsRNA Contained in a Virus-Like Particle?

The possibility of the dsRNA associated with hypovirulence being packaged in a virus-like particle (VLP) is not unlikely and has been suggested by many workers. Such a VLP has been reported by Dodds (1980a) and Chmelo (this proceedings). Dodds (1980a) has isolated a club-shaped particle from *E. parasitica* strain 113 which he believed packages the dsRNA within the fungal cell. This pleomorphic club-shaped particle can be extracted with polyethylene glycol and purified by differential, then cesium chloride (CsCl) equilibrium density centrifugation. In our lab, we (Hansen, Gillies and Van Alfen, unpublished data) have confirmed Dodds' observations concerning the fact that the crude preparation of pleomorphic particles can be separated into two peaks from a CsCl gradient. The denser of the two (1.27 g/cm^3) contains the dsRNA. We have analyzed the composition of these particles and determined that they are very unlike a typical virus. There is an unusual amount of carbohydrate present. In fact, it comprises the greatest proportion of the particle. Gas chromatography analyses by the alditol-acetate method (Jones and Albersheim 1972) show the same sugars are present in the particle as those found in the fungal cell wall. Radiolabeling experiments show several different proteins are present, but not one in an amount sufficient to be a capsid. Another peculiarity about this so-called VLP is that of those strains of *E. parasitica* examined thus far, only EP-113 and EP-43 (Chmelo this proceedings) are found to contain the particles. Thus, it is

not clear whether this particle is actually a virus or something else, e.g. a fungal vesicle used to isolate the dsRNA or perhaps an artifact of the purification method.

Areas in need of future investigation are clearly evident. How is this club-shaped particle formed? If it is a virus, how and where does it replicate? Are there polymerases present? Does it have a capsid and how is dsRNA packaged within the virion? In addition, how is it related to other mycoviruses? If it is not a virus, then what is the purpose of the particle which contains dsRNA? The fact that the particle composition is similar to the cell wall may indicate that the membranes are a mode of removing dsRNA from the fungal cells. Transmission electron micrographs show VLP's associated with the endoplasmic reticulum of a hypovirulent strain, yet absent from a virulent one (Newhouse et al. this proceedings). The endoplasmic reticulum is the site of cell wall carbohydrate synthesis. This may explain the similarity between sugar composition of the pleomorphic dsRNA containing membrane-bound particles and the cell wall. These particles may be vesicles associated with cell wall synthesis. The knowledge of how the dsRNA is packaged within the fungal cell will undoubtedly help us understand variables involved in cell to cell transmission of the dsRNA.

What is the Nature of the dsRNA of TH Strains?

The dsRNA associated with hypovirulence, while the subject of much speculation, has been poorly characterized. The studies that have been completed on electrophoretic banding patterns and dsRNA size are a good beginning at characterizing the dsRNA, but they are only a beginning. We currently know that the dsRNA of different TH isolates may differ greatly in their electrophoretic banding patterns (Dodds 1980b). These banding patterns change at times, however, upon transfer to new V strains or upon subculturing (Anagnostakis and Day 1979). Such variability in the composition of dsRNA segments is not typical of the dsRNA of mycoviruses (Buck 1980). The dsRNA segments of known mycoviruses are quite stable in their electrophoretic banding patterns. Thus, we need to determine whether this variability represents mixed infections of different mycoviruses, or whether deletions from individual dsRNA segments are a frequent occurrence. A similar complex and variable segment pattern in *Gaeumannomyces graminis* var. *tritici* was explained on the basis of mixed infections (Buck et al. 1981). In the case of TH of *E. parasitica* such mixed infections may not be the explanation since dsRNA containing virulent strains have not been found (Elliston 1978). We would expect that not all dsRNA segments would cause the hypovirulence phenotype, thus mixed infections would be expected to result in the occurence of dsRNA containing V strains. Deletions in dsRNA virus genomes have been recorded, but they serve as poor models for what is occurring in the dsRNA of *E. parasitica* since deletions are a rare event (Rubenstein and Harley 1978).

Probably the most direct approach to studying the relationship between dsRNA segments found in *E. parasitica* is to determine the relatedness of the different segments to one another. Such studies can be done using hybridization techniques or nucleotide mapping. The study of the homology between the dsRNA segments would also answer questions of whether there are great differences between the dsRNA of European and North American TH strains. If

the dsRNA of the European and American TH strains is different then we must seriously consider the possibility that there are different TH factors.

In addition to homology studies, better physical characterization of the dsRNA is needed. Currently the double-strandedness of the RNA is known by behavior on cellulose columns and by selective degradation by enzymes. Physical evidence such as melting to the predicted molecular weights of the single strands is needed to confirm the double-strandedness of the RNA. Molecular weights also need to be confirmed by physical methods. Currently the molecular weights are estimates based upon comparative electrophoresis with standards (Dodds 1980b). Unfortunately the largest dsRNA segments from *E. parasitica* are at the limit of judicious use of standards.

How does the dsRNA Regulate Virulence of *Endothia parasitica*?

Virulence reduction in *E. parasitica* induced by dsRNA has no models in biology to draw upon for comparison. We currently know nothing of how virulence is modulated by the dsRNA so all we can do in this paper is speculate. There are at least three possible general mechanisms whereby the dsRNA could modulate virulence. One would be to compete by replication for critical cellular metabolites. Since most mycoviruses are latent, having no discernible effects on the fungus, replication of a mycovirus does not appear to adversely affect most fungi. However, the competition for a specific metabolite important in virulence may have an effect on virulence. Virulence could also be reduced by the effect of a translational product of the dsRNA at some metabolic control pathway critical to virulence expression. A third possibility is that the dsRNA directly interacts with the fungal genome at a virulence control locus. The existence of such a locus is suggested by the high frequency appearance of non-transmissible hypovirulent isolates after UV irradiation (Van Alfen et al. 1978b) or osmotic shock (Van Alfen and Hansen this proceedings). These non-TH isolates are phenotypically identical to TH isolates, but they lack dsRNA (Van Alfen and Hansen unpublished data). These isolates may represent a class of control site mutants.

The problem of determining how virulence is affected by the dsRNA will not be easily solved. The question does have much appeal so hopefully many researchers will be attracted to the problem to ensure progress to its solution. If a mechanism is found that is exploitable in other pathogens, the effort expended on this research could result in wide application.

Conclusion

The research questions posed in this discussion are certainly not comprehensive, nor will their solution provide more than a good beginning toward understanding the phenomenon of hypovirulence of *E. parasitica*. Progress must be made toward their solution, however, if we are to determine the potential of this system for wider exploitation. We must also learn more of the biological nature of the TH factor so that some of the problems encountered in its field use for control of chestnut blight can be overcome. Such knowledge would allow intelligent planning rather than wild guessing to direct our research efforts. Without an understanding of the properties of the TH factor we are no better able to exploit it for control of chestnut blight than we can repair our own laboratory instruments. We must have an

understanding of how our instruments work before we can repair them. Likewise we must have an understanding of the properties of the TH factor before we can optimize its spread.

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CULTURAL CONSIDERATIONS IN ENDOTHIA PARASITICA; STATE OF THE ART

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ABSTRACT.--*Factors affecting in vitro growth, reproduction, and pigment formation by Endothia parasitica are discussed from a historical perspective. There also is a brief discussion on the methods currently used for sexual reproduction studies, vegetative compatibility, and conversion testing. The potential for the need of a selective medium for use in dissemination studies also is mentioned.*

The purpose of this "State of the Art" paper is to renew an awareness of factors that need to be considered when studying the cultural behavior of *Endothia parasitica*. This paper is not a review of all the literature on *in vitro* growth, reproduction, pigment formation, etc., of this fungus. Rather it is a discussion, from a historical point of view where possible, of some of the cultural and environmental factors that have been shown to affect *E. parasitica* and how this relates to present-day research. Until recently, all research was conducted with what are now considered normal-virulent isolates of this fungus; hypovirulent isolates are "abnormal" culturally. Therefore, the results cited from work done by the early workers are confounded by hypovirulence.

Even though the topics discussed in this paper are interrelated, they are arbitrarily divided. Part of the discussion will be centered on the affect of nutritional and environmental factors on cultural behavior, especially the formation of pigment and pycnidia. There also will be a brief discussion of the kind of pigments produced by this fungus and the current *in vitro* methods used for sexual reproduction studies and vegetative compatibility and conversion testing. There also will be a brief mention for the need of a selective medium for use in dissemination studies, especially as related to hypovirulent isolates.

Cultural Characterics

General medium effects

Endothia parasitica grows readily *in vitro* and on a variety of substrates. This lack of fastidious growth requirements has advantages and disadvantages.

It is an advantage because the fungus grows so readily on such a variety of substrates, the choice of substrate is left to the researcher. Anderson and Rankin (1914) noted in the saprophytic condition this fungus seemed omnivorous. This lack of fastidiousness is a disadvantage, however, because little definitive work on the basic physiology of this organism has been conducted.

Early workers noted *E. parasitica* grew and sporulated on a large variety of substrates: sterilized root and twigs of a variety of tree and shrub species, bean plugs, carrots, potatoes, sweet potatoes, bread, corn meal, oat meal, rice, sugar solutions, bouillon, lima bean agar, oat agar, potato agar, corn meal agar, chestnut bark media, prune agar, starch, and Raulin's fluid to list a few (Anderson and Babcock 1913; Anderson and Rankin 1914; Clinton 1909; 1912a; 1912b; Cook and Wilson 1914; Heald et al. 1915; Shear and Stevens 1913; Shear et al. 1917). Yet with the tremendous diversity of substrates suitable for supporting growth of this fungus, relatively few have been used extensively and the cultural behavior of this fungus has been described in detail on still fewer. Much of the early cultural work was conducted in an effort to develop reliable cultural criteria that could be used to distinguish *E. parasitica* from several closely related *Endothia* species (Anderson and Anderson 1913); Clinton 1912a; Shear and Stevens 1913; Shear et al. 1917). These cultural parameters were especially valuable when the sexual stage of the species in question was not available (Shear et al. 1917).

A few of the culture media used by these early workers and a description of the cultural characters observed are going to be mentioned. Although the media they used may no longer be important, the types of cultural characteristics and their variability as affected by the test medium as well as the detailed observations of the cultural morphologies made are relevant to current work comparing normal and hypovirulent *E. parasitica* isolates.

Murrell commented (quoted in Anderson and Rankin 1914) that when *Diaporthe (Endothia) parasitica* was "grown in artificial culture, the mycelium is at first pure white, changing to a yellow with age and the fruiting pustules are a beautiful yellow." This description still is accurate for normal isolates of this fungus grown on some media. Anderson (1914) reported that "on potato agar the mycelium begins to turn yellow due to the production of pigment in the cells at the end of 4 to 6 days." "As the cultures age, the fungus often becomes purple or wine colored." Shear and Stevens (1913) reported when pycnospores of *E. parasitica* were streaked on a potato agar they described, (not the same medium used by Anderson 1913) "at the end of 3 or 4 days at room temperature, there was short, fluffy, white aerial growth along the streak. The surface of the mycelium was orange by transmitted light while by reflected light, it was between raw sienna and antique brown at the sides. Within 6 days the mycelium, especially at the base of the agar slant, took on a peculiar granular metallic appearance, due apparently, in part at least, to the character of the mycelium and in part to the minute water drops scattered over the surface. This portion of the culture was light orange-yellow by reflected light and orange by transmitted light. The peculiar surface appearance might perhaps be called "brassy". This metallic surface appearance has been found to be the most constant and reliable distinguishing character of *E. parasitica* on potato agar. In 12 to 14 days, small pycnidial pustules appeared in the upper portion of the tubes, and the agar just below the mycelium became warbler green." When grown on corn meal agar in Petri plates, these same authors (Shear and Stevens 1913) noted that "1-week-old cultures of *E. parasitica* covered about one-half the surface of

the medium. The outer margin was pure white, the remainder buff yellow, with a superficial white growth above and the medium uncolored. A few small pustules with spore masses occurred near the point of inoculation. Cultures 1 month old showed a compact growth nearly smooth in the surface. The superficial mycelium was pale yellow. The pale yellow-ocher spore masses were minute, very numerous and nearly covered the surface. The medium was slightly greenish about the sides of the flask just beneath the mycelium." The number and size of the pycnidia produced on this medium was considered to be an important character for distinguishing *E. parasitica* from other *Endothia* species (Shear and Stevens 1913). Clinton (1912a) also noted a yellow pigment developed on a lima bean medium but as the culture aged, the pustules became light chestnut brown. Later Shear et al. (1917) reported on additional cultural studies with species of *Endothia*. They noted the cultural characteristics produced by *E. parasitica* on corn meal agar in unslanted tubes were very reliable for distinguishing it from other *Endothia* species. They reported that after 6 to 8 weeks, there was "a scantly white growth of superficial mycelium, with several prominent pycnidial pustules clustered near the center area of a slightly darker shade than the raw sienna." In this same paper, they (Shear et al. 1917) also reported on the cultural characteristics of *E. parasitica* on a semi-defined, liquid medium, referred to modified Cooks medium No. II. After 1 month of incubation in 30 ml of this media in 100 ml Erlenmeyer flasks, the mycelial growth was "very abundant, closely matted, chiefly submerged but slightly aborescent in one or two small areas which remained above the surface." The color of the mycelium was a dark greenish brown (Shear et al. 1917).

Although descriptions of cultural characters on various substrates could continue; these examples demonstrate that the substrate affects cultural morphology and pigment formation in this fungus. Rather than being concerned about characterizing species of *Endothia*, today we are more concerned with the cultural variability that exists between and among normal and hypovirulent isolates of *E. parasitica* and how the chosen substrate affects this variability. Most workers in North America currently use Difco potato dextrose agar for these types of comparisons. Workers in Europe often use other media including Maltea-Moser agar. These media work well for distinguishing normal from hypovirulent isolates, but it is not always possible to make direct comparisons between European and North American work. In addition, if a potato dextrose agar medium other than Difco is used, cultural characters often are drastically altered. Even though there are culture media that work very satisfactorily for distinguishing between normal and hypovirulent isolates and among hypovirulent types, defined media should be developed that discriminate among these isolate types. This would improve repeatibility of results among workers and might provide information on the physiological differences that exist among these isolate types.

Pigment and pycnidia formation

In addition to the general affects the medium constituents have on the cultural behavior of *E. parasitica* and how this behavior is confounded by hypovirulence, factors affecting *in vitro* pigment and pycnidia formation warrant further consideration. The production of pigment and asexual reproduction are closely associated in this fungus. The characteristic yellow-to-orange color produced by normal isolates of this fungus on many media is thought to be similar to that responsible for the characteristic color of the stromata

in the bark (Anderson 1914). Even if the medium or the environmental conditions do not support intensive *in vitro* pigment formation, if pycnidia are produced, they usually are pigmented yellow-to-orange (Shear et al. 1917). Since the lack of pigment and normal pycnidia formation on Difco potato dextrose agar or Maltea-Moser agar is one *in vitro* criterion used to distinguish some hypovirulent isolate types from normal ones, the cultural, physiological, environmental, and biochemical factors affecting pigment formation and reproduction in this fungus need further study. In addition, when one considers there are hypovirulent isolates (the JR and North American types) that produce abundant pigment on Difco potato dextrose agar but do not produce normal pycnidia, the significance of understanding the mechanism of pigment formation and its association with the development of pycnidia is further reinforced.

Pigment formation. There was considerable interest in pigment formation by species of *Endothia* on various media by early workers. Some of this work regarding the affect of the medium on pigment formation already has been mentioned, so additional comments will be restricted to the types of pigments produced by *E. parasitica*. Pantanelli (in Anderson 1914 and Roane and Stipes 1978) noted the superficial mycelium in culture was bright yellow because of lipochrome drops in the cells. Later Anderson (1914) suggested the pigment was aurine rather than a lipochrome. He observed that as the cultures of *E. parasitica* aged on a potato agar, the pigment changed from a yellow to a purple. He related this change in color to change in the pH of the medium, acidic to alkaline. The production of pigment on a corn meal medium was one cultural criterion that could be used to separate *E. parasitica* from other closely related *Endothia* species (Shear et al. 1917). Hawkins and Stevens (1917) described pigments produced by three species of *Endothia* and concluded they probably were not aurine or lipochrome. Sands (1919) examined one of these pigments in detail. He assigned it the emperical formula $C_7H_5O_4$, concluded it was probably related to members of the pyrocatechin group, and named it endothine red. It was not until Shibata et al. (1953; 1955a; 1955b) began working with the pigments of *Endothia* species, however, that details of their chemistry were unraveled. Four pigments, skyrin, skyrinol, oxyskyrin, and rugulosin were identified in species of *Endothia* (Shibata et al. 1953; 1955a; 1955b). Skyrin, oxyskyrin, and sykrinol are 1,1-bisanthraquinones whereas rugulosin is a modified bisanthraquinone (Shibata 1967; 1973). Shibata (1967) developed a biogenetic-scheme for the production of these pigments from acetyl and malonyl coA. Production of secondary metabolites by the condensation of an acetyl unit with malonyl units is characteristic of fungi and often results in formation of pigments (Turner 1976). The metabolism of acetate by *E. parasitica* has been shown to shift towards the synthesis of bisanthraquinones and fatty acids during sporulation (McDowell and DeHertogh 1968).

Roane and Stipes (1978) reported skyrin, oxyskyrin and rugulosin were present in cultures of *E. parasitica* grown on a white corn meal medium. The one hypovirulent isolate they tested, EP-43, also contained these pigments. With the increased number of hypovirulent isolates that have been found, the constantly improving biochemical techniques, and the variation in pigment formation in culture among these isolates, additional work on the types of pigments present and their variation, if present, between and among normal and hypovirulent isolates of this fungus warrants further work. The results of such studies might provide additional criteria for distinguishing between

and among normal and hypovirulent isolate types and information on the metabolic and biochemical disturbances caused by hypovirulence.

Pycnidia formation-light effects. Light also has been shown to affect pycnidia and pigment formation in this fungus. Anderson (1914) stated "when plate cultures are grown in total darkness on chestnut agar, no pycnidia are developed, while on plates made at the same time and grown in the light, the usual rings of pycnidia appear." When cultures were incubated in darkness until the medium was about half-covered with mycelium and then exposed to light, "circles of pycnidia were developed, beginning with the ring which marked the outermost limit of the colony when removed from the dark chamber. The concentric rings which always appear on agar cultures are due to the alternation of night and day" (Anderson 1914). Yet, Shear et al. (1917) noted "it is evident that pycnidia are produced abundantly in total darkness on chestnut-twig agar as well as on other favorable media. There is no perceptible difference in amount of spore production or in the arrangement of pycnidia between cultures kept in total darkness and those kept in light during the day if the temperature and evaporation remain the same in both."

Leonian (1924) also reported light did not affect pycnidia formation on a glucose-malt extract-peptone medium. In contrast, Barnett and Lilly (1952) demonstrated pycnidia formation on a glucose-casein hydrolysate agar medium was influenced by the wave length, intensity and length of exposure to light. Total darkness decreased the number of pycnidia produced, but those formed were larger than those in continuous light. White to blue (400 to 500 um) light were most stimulatory for pycnidia formation. A few large pcynidia were produced in red light, similar in number and size to those produced in darkness, and yellow and green light had an intermediate effect (Barnett and Lilly 1952). Campbell (1967), in the most critical recent study on nutritional factors affecting *in vitro* sporulation, used a carefully prescribed light treatment. After the plates were inoculated, they were wrapped in two layers of aluminum foil and incubated at 25 C for 5 days. The cultures were then exposed for 15 minutes to fluorescent lamps (130 fc, Champion cool-white with emission peaks in the blue region of the visible spectrum). After 3 days of additional incubation in darkness, sporulation was evaluated. Light also has been shown to enhance sporulation on a Difco potato dextrose agar medium (Anagnostakis 1978), and Grente and Sauret (1978a; 1978b) reported that pycnidia production by normal isolates on a Maltea-Moser agar began on the third day when cultures were grown in alternating light and dark. A few hours of light were enough to initiate pycnidia formation on this medium. In continuous darkness, pycnidia were thinly distributed and only appeared after 7 days' growth. Pycnidia were formed after 15 to 20 days by white, hypovirulent isolates if the culture was exposed to light more than 50 per cent of the time. Only a few pycnidia formed and there was no distribution pattern present (Grente and Sauret 1978a; 1978b).

These results demonstrate light stimulates *in vitro* pycnidia formation, at least on some media, and that normal and hypovirulent isolates respond differently. The effect of light on pycnidia (and pigment) formation needs additional work, and the wave length, intensity, duration, etc., of the light should be closely examined. There are means to examine the effects of light on fungi much more critically than what has been reported with *E. parasitica* (Trione and Leach 1969; Tan 1978).

Pycnidia formation-nutritional effects. Leonian (1924) also reported that *E. parasitica* could be induced to produce pycnidia by manipulating the nutritional conditions of the medium. He used a technique with several fungi, which consisted of growing the fungus in a medium (glucose-malt extract-peptone for *E. parasitica*) that was suitable for vigorous vegetative growth. The mycelium was then aseptically transferred to a weak medium. Often pycnidia formation was stimulated by these fungi when transferred to the weak medium, or starvation conditions. With *E. parasitica*, however, only pycnidial initials formed when the mycelium was starved, but if this mycelium was placed in a favorable medium again, optimal sporulation occurred. More recently, Campbell (1967) demonstrated the concentration of the carbon source and the nitrogen source as well as the method used to sterilize the medium affected pycnidia formation. The carbon to nitrogen ratio did not affect sporulation within the ranges he tested, but the absolute qualities and the type of carbon and nitrogen used, determined the intensity of sporulation. The degree of utilization of a carbon source, especially fructose, was affected by the method of medium sterilization. He found pycnidial formation was optimal in the medium containing fructose as the carbon source, if the entire medium was filter sterilized, or if fructose was sterilized separate from the other medium constituents, either by autoclaving or by membrane filtration. Certainly additional work on the nutritional basis for pigment and pycnidia formation is justified. The results of this work and that with the effect of light, etc., should provide for a more complete understanding of the factors affecting pigment and pycnidia formation and how hypovirulence affects their formation.

Sexual Reproduction

Techniques have been developed that support *in vitro* sexual reproduction by *E. parasitica* (Anagnostakis 1979; Willey 1980). It is now possible to perform controlled crosses so studies can be conducted on the inheritance of genetic traits in this fungus. In addition, test crosses can be conducted between normal and hypovirulent isolates. Although such crosses have been attempted unsuccessfully, additional studies on the nutritional and environmental (light, temperature, etc.) factors that affect *in vitro* perithecia formation can now be conducted.

Vegetative Compatibility and Conversion

Vegetative incompatibility among *E. parasitica* isolates may present a serious problem for application of hypovirulence. Transfer of hypovirulence factors between normal and hypovirulent isolates requires hyphal anastomosis and cytoplasmic exchange between the isolates. Vegetative incompatibility apparently prevents the exchange of cytoplasm and results in the failure of some hypovirulent strains to cure virulent cankers (Anagnostakis 1978). The vegetative compatibility (v-c) type of normal isolates can be determined using the technique described by Anagnostakis (1977; 1978). The medium (Difco potato dextrose agar), cultural conditions, age of the inoculum, placement of the inoculum on the test media, etc., as described by Anagnostakis must be closely followed for reliable results. If there was a defined media that would work as well or better, the problem of variation in natural media could be eliminated and results among workers would be more comparable. A defined media, if there was one that would work well for discriminating between normal and hypovirulent isolates, also might function for v-c testing.

Conversion also will occur when compatible, normal and hypovirulent isolates are paired on agar media (Grente and Sauret 1978a; Northup 1981; Anagnostakis 1982). Conversion does not occur as readily between incompatible isolates. Grente and Sauret (1978a) suggest conversion does not occur here because anastomoses between incompatible isolates results in degeneration of the cytoplasm at the site of the hyphae fusion, and this prevents complete cytoplasmic exchange. Northup (1981) developed a defined medium and a technique for microscopic examination of individual anastomosis between isolates. Hyphae to peg and peg to peg types of anastomoses (Buller 1933) were observed to occur between both vegetatively compatible and incompatible isolates. Collapsed cells, observed with both light and scanning electron microscopy, were abundant in the hyphae immediately adjacent to the site of hyphal anastomosis between incompatible isolates. Similar shrunken cells also were noted by Anagnostakis (1982). Whether the abnormal appearance of these cells is associated with cytoplasmic degeneration which may prevent transmission of hypovirulence between incompatible normal and hypovirulent isolates is not known. Microscopic examination of hyphal anastomosis in this fungus needs further attention. There are media and techniques available now that are suitable for use in these types of studies (Northup 1981; Anagnostakis 1982). The results of these types of tests should provide for more basic understandings of the nature and control of the transmission of hypovirulence in this fungus.

Selective Medium

One of the questions facing people working with hypovirulence as a biocontrol for chestnut blight is how are hypovirulent isolates naturally disseminated. These isolates often do not produce many pycnidia in host bark and there is no evidence they produce perithecia in nature. Natural dissemination has occurred in Italy (Turchetti 1978), however, and is occurring in Michigan. Natural dissemination of artificially introduced hypovirulent isolates also has been reported in West Virginia (Willey this proceedings). Workers have suggested birds, various insects, and mites may function as carriers for this fungus (Anderson and Babcock 1913) and might effectively carry hypovirulent isolates within and among trees (Anagnostakis this proceedings; Wendt et al. this proceedings). *Endothia parasitica* spores have been washed from insects that were observed to visit cankers. But, however unlikely, there may be an air-borne propagule that is important for natural dissemination of hypovirulent isolates. In order to determine if such a propagule exists, the air must be sampled in areas where hypovirulent isolates are spreading naturally, Italy, Michigan, and West Virginia. There are numerous methods for sampling the air, but because of the tremendous diversity and number of fungal spores that probably would be encountered, it might be difficult to microscopically examine for the fungus directly, and it might be difficult to culture the fungus from the sampling device. To facilitate air sampling studies, the use of a medium that is selective for *E. parasitica*, especially hypovirulent types, might be helpful. Anderson and Babcock (1913) and Heald et al. (1917) used petri plates with agar media to sample for air-borne *E. parasitica* spores. The medium used by Heald et al. (1917) was selective. It inhibited growth of bacteria, retarded growth of fast growing fungi, and allowed for good growth and easy identification of the chestnut blight fungus. The results of this work clearly demonstrated ascospores were present in the air in blighted chestnut stands, and these workers concluded ascospores were responsible for long-distance dissemination of this

fungus (Anderson and Babcock 1913; Heald et al. 1917). There are no other reports of the use of selected media until the recent report by Russin et al. (this proceedings). Until there is conclusive data explaining the mechanism of natural dissemination of hypovirulence, researchers must not rule out the possibility of some form of aerial dissemination, and the use of a selective medium might be valuable for such studies.

There probably are other cultural considerations that should have been addressed in this paper. However, if the reader has become only slightly more aware of the lack of information and understanding of the cultural behavior of *E. parasitica*, then the author's purpose will have been fulfilled. With the discovery of hypovirulence in this fungus and the unexplained cultural and pathogenic abnormalities caused by the hypovirulence factors, the lack of basic information is confounded. Challenges still remain therefore, for those interested in studying the cultural behavior of *E. parasitica* and how hypovirulence affects this behavior.

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VEGETATIVE COMPATIBILITY AND HYPOVIRULENCE CONVERSION IN *ENDOTHIA PARASITICA*: STATE OF THE ART

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ABSTRACT.--*This paper reviews the recent work on vegetative compatibility (v-c) in Endothia parasitica and relates v-c to hypovirulence conversion. Endothia parasitica has at least 128 v-c groups but hypovirulence conversion is not as limited as vegetative compatibility. For example, seven isolates with hypovirulence from four v-c groups converted isolates from 38 of 48 v-c groups tested. Recent evidence indicates that at least some alleles for v-c have a quantitative rather than a qualitative effect.*

Vegetative compatibility (v-c) refers to the characteristic enabling two hyphae to make contact, fuse, and exchange cytoplasm or nuclear material. Often the negative response, vegetative incompatibility, is used because incompatibility occurs more commonly than compatibility. In *Endothia parasitica*, hypovirulence (H) due to a cytoplasmic factor is spread by hyphal fusion and cytoplasmic exchange. In many ascomycetes, heterokaryon compatibility rather than vegetative compatibility is used to describe the portion of the parasexual cycle in which hyphal fusion results in two or more genetically different nuclei in a vegetative hypha. Heterokaryons of *E. parasitica* can form when compatible isolates of normal virulence are paired but never form from pairing hypovirulent isolates (Anagnostakis 1981; Puhalla and Anagnostakis 1971; Van Alfen et al. 1975). Puhalla and Anagnostakis (1971) found heterokaryons formed in pairings between virulent, auxotrophic mutants on minimal media but conidia from the heterokaryons infrequently were prototrophic. Thus, the mycelium maintained the heterokaryotic condition but the conidia were generally uninucleate and ended the condition. Van Alfen et al. (1975) used auxotrophic mutants to demonstrate that H was transferred from a hypovirulent isolate to a virulent isolate. Paired inoculations of a H, lysine, auxotroph and a virulent, methionine auxotroph yielded only H, methionine auxotrophs from two restricted cankers. Besides indicating that the H factor was extrachromosomal, this sample suggested heterokaryosis had not occurred. Subsequently, Anagnostakis (1981a) reported heterokaryons never resulted from pairings of virulent and H isolates in culture or on the host even when auxotrophs produced from the same original isolate were used. Apparently, the addition of the H factor in the cytoplasm has changed the capacity of the hyphae to maintain the heterokaryon even in culture. Anagnostakis (1981b) has also reported the infrequent occurrence (1:36 isolates) of a heterokaryon in nature. Since heterokaryons do not form in pairings of H and virulent isolates, there is

less chance for the parasexual cycle to initiate new genetic combinations to possibly offset the effect of H. The normal sexual cycle offers greater probability of new genetic combinations than does the parasexual cycle. However, Elliston (1978) indicated H strains produced perithecia infrequently compared to virulent strains. Genetic integrity seems to be clearly maintained in *E. parasitica* as will be indicated again later.

A technique developed by Anagnostakis (1977) has proven useful in determining v-c groups among virulent isolates of *E. parasitica*. This technique took advantage of observations made by Andes (1961) and Rizet (1952). Vegetative incompatibility in *Podospora anserina* was indicated by a clear zone between colonies because hyphae died back to septa after anastomosis. Perithecia formed on each side of the clear zone in what was called a barrage, provided that the isolates were of opposite mating type (Rizet 1952). Three types of response between single ascospore isolates of *E. parasitica* were a clear zone of inhibition, a line of pycnidia, and a smooth merging of mycelia (Andes 1961). Anagnostakis (1977) made pairings of *E. parasitica* isolates on Difco potato dextrose agar with methionine and biotin (dPDAmb) and placed the dishes in the dark at 25 C for 5 to 10 days. Pairs either had a complete merging of mycelia or formed a row or rows of pycnidia (a barrage). Merging pairs were classified as being from the same v-c group whereas presence of barrage indicated different v-c groups. Initially, 28 v-c groups were identified among isolates from Italy, France, and the United States (Anagnostakis 1977). Later, when 77 different v-c groups were identified, Anagnostakis (1980) indicated there would need to be alleles at least at seven loci to produce that number of groups and 2^7 could code for 128 possible v-c groups.

The larger the number of v-c groups present in a canker or in a forested area, the more difficult it would seem to be to control the disease by maintaining a population of compatible H isolates. We have used the Anagnostakis method to determine the number of v-c groups present in individual cankers and among cankers on the same tree and in small groves. Individual cankers often have two or more v-c groups present. The isolated tree at Bonair, Tennessee, which yielded 35 percent H isolates of the total *E. parasitica* isolates present (Kuhlman this proceedings), had four v-c groups present in the six cankers. One canker had all four groups, whereas four cankers had three groups, and one had two. The 41 cankers from near Buchanan, Virginia, (Kuhlman this proceedings), yielded an average of 2.3 v-c groups per canker. We are still determining the number of distinct v-c groups among the 93 groups identified in individual cankers, but estimate 12 to 15 groups present in the area of approximately an acre.

Both the incompatible (barrage) response and the compatible (merge) response vary in discreteness among isolates. Most isolates merge only with a few isolates in a typical response; others merge with isolates from several v-c groups, whereas a few isolates are incompatible even with themselves. This suggested that some isolates contained either hyphae or nuclei from several v-c groups. Ten hyphal tip isolates were made from each of five isolates with typical, broad, or incompatible responses. The ten hyphal tip isolates within each of the 15 parent isolates were paired with each other and all were compatible with all other propagules within the family. Anagnostakis (1977) also reported no segregation for v-c groups from single conidial isolates of either virulent or H isolates.

The merge response is typical when isolates are selfed, whereas the barrage is understood to be a response to incompatibility and failure to establish cytoplasmic exchange. Anagnostakis (1977) recognized the relationship of v-c grouping and conversion of virulent isolates by H isolates was not directly related since virulent isolates from 13 v-c groups were controlled by H isolates from 3 v-c groups in field experiments. A laboratory method for determining conversion capacity of H isolates utilizes growth of H and virulent isolates on a cellophane film over dPDAmb (Anagnostakis and Day 1979). At 28 C on a 16-hour day, conversion may occur within 2 days and up to 8 days. The cellophane film increases the probability of hyphal contact between the two isolates; however, in our lab, conversion is frequent on dPDAmb at room temperature (19 to 24 C) without cellophane. Under these conditions, conversion is apparent after 4 to 10 days of growth.

Anagnostakis and Day (1979) paired seven H isolates from four v-c groups with virulent isolates from 48 v-c groups. Their data are presented as the response of the virulent isolate to the seven H isolates (Table 1). Twenty-three of the 38 v-c groups that were converted were converted by H isolates

Table 1. Data from Anagnostakis and Day (1979) showing conversion (C) of virulent isolates from 48 v-c groups by seven H isolates from four v-c groups

Virulent isolates: v-c group number(s)	H isolate: v-c group:	113 10	4b 10	A 17	B 38	5A 40	5B 40	5C 40
40		C	C	C	C	C	C	C
17		C	C	C		C	C	C
38		C	C		C	C	C	C
10		C	C			C	C	C
26		C	C				C	C
21			C		C	C		C
36				C		C	C	C
3, 32		C	C		C		C	
48			C		C		C	C
8, 18		C	C		C			
12		C	C	C				
43					C	C	C	
19, 37			C				C	C
28			C		C	C		
34			C	C	C			
31							C	C
7		C	C					
50					C		C	
41, 45, 46			C				C	
4			C		C			
25, 27		C						
1, 6, 9, 22			C					
23					C			
15						C		
39, 42, 47, 49							C	
20								C
2, 5, 11, 14, 16, 29, 30, 33, 35, 44								

from two or more v-c groups. Anagnostakis and Day (1979) said that "hypo virulence conversion capability" was less restrictive than v-c of normal isolates. On the other hand, conversion by different isolates from v-c groups 10 and 40 varied considerably even though isolates within each group were from the same parent isolates. Although 27 v-c groups were converted by H isolates from v-c 10, only 11 were converted by both isolates of group 10. Similarly, only 5 of 25 v-c groups that were converted by v-c 40 isolates were affected by all three isolates.

Virulent isolates within a v-c group also vary in response to H isolates. Data from Bonair, Tennessee, are presented in Table 2. Three to five isolates from the four v-c groups were paired with 13 H isolates. In each v-c group, some isolates were not converted by H isolates that converted other

Table 2. Conversion (C) of virulent isolates from four v-c groups by 13 H isolates from the same American chestnut source near Bonair, Tennessee

Virulent isolate	v-c group	H isolate[a/] 1-8	1-15	2-11	2-13	3-8	3-20	4-7	4-12	5-2	5-11	6-1	6-5	6-19
1-13	A			C			C							
2-14	A			C										
6-2	A			C			C							
3-9	D						C							
4-5	D						C						C	
5-7	D						C							
6-18	D												C	
1-12	E	C								C				C
2-3	E	C		C			C			C				C
4-31	E	C		C			C			C				C
5-4	E	C								C				C
6-4	E	C					C			C				C
1-2	B		C	C	C	C			C		C	C		
2-27	B		C		C	C			C		C	C		
3-17	B				C	C			C		C	C		
4-16	B		C		C	C			C		C	C		
5-13	B			C	C	C			C					
6-14	B		C		C	C			C		C	C		

[a/] Isolate number = canker number - chip number.

isolates within the group. Hypovirulent isolates 2-11 and 3-20 converted virulent isolates in three v-c groups. These data indicate that grouping isolates by v-c groups is helpful in terms of conversion potential but exceptions to conversion are not unusual either positively or negatively.

The discrepancy between v-c groups among virulent isolates and conversion of virulent isolates within and among v-c groups by H isolates may be explained in several ways. One likely explanation is that conversion requires only minute infection of H cytoplasm into the virulent cytoplasm. Field data indicate conidia carry enough of the H factor to provide control of treated

cankers (Kuhlman this proceedings). In laboratory tests, hyphal contact needs only permit exchange of a single dsRNA particle to establish conversion so that disruption of the cytoplasmic exchange after hyphal walls are dissolved seemingly would not prevent conversion.

Anagnostakis (1980) explained the discrepancy from a genetic point of view. Matings of isolates from some v-c groups yielded progeny of only the parent v-c groups, suggesting that only one allele segregates. Some matings yielded progeny of four v-c groups indicating alleles at two loci were different. Some parent isolates have different alleles for compatibility at four loci and the progeny should represent 16 (2^4) v-c groups. If we presume the seven alleles that Anagnostakis (1980) has identified function in various portions of plasmogamy, i.e. hyphal contact, cell-wall breakdown, cytoplasmic exchange, etc., each allele may function in one step of this process. If several alleles are the same in two isolates, plasmogamy may proceed far enough to permit exchange of the H factor even though it is subsequently disrupted by an incompatibility in gene function.

Conversion of virulent isolates by H isolates is affected quantitatively by the number of genes that differ for compatibility (Anagnostakis and Waggoner 1981). Anagnostakis and Waggoner (1981) paired virulent and H isolates which differed by 0, 1, 2, and 5 v-c genes. The fewer genes different, the smaller the canker development on American chestnut. Pairs that differed by five v-c genes formed cankers which were not significantly different in size from those formed by two virulent isolates from the same v-c groups. Although Anagnostakis and Waggoner (1981) suggest their results are similar to Caten's (1972; 1973) results with *Aspergillus amstelodami*, they apparently differ in regard to the frequency of occurrence of quantitative alleles. Caten (1972) initially stated that transfer of vegetative death, a cytoplasmically inherited trait, between strains of *A. amstelodami* was completely prevented if strains differed by more than one gene. Later, Caten (1973) reported the discovery of a gene locus that had a quantitative rather than qualitative effect. This locus restricted but did not abolish heterokaryon formation. Four other gene locations acted qualitatively with any one individually determining incompatibility. Further genetic work will be needed to determine if Anagnostakis and Waggoner's (1981) conclusions regarding the additive nature of the v-c genes in *E. parasitica* are true for all gene pairs or only for a few of the seven involved. The long-term effects on canker development and tree survival of quantitative versus qualitative traits also need to be evaluated to be certain disease control is achieved.

Vegetative compatibility grouping is helpful in establishing possible relationships among virulent isolates but conversion of virulent isolates by H isolates is necessary to confirm the relationships. A definitive test for conversion and thus for vegetative compatibility should include: pairing of virulent and H isolates, noting changes in virulent colony morphology that suggests conversion, transferring the converted isolate, retention of H morphology by the transfer, and conversion of the normal isolate by the converted transfer. These steps are necessary because isolates often seem to change their growth habit when confronted by another isolate. By subculturing the apparent convert, the permanence of the change can be confirmed. At least, occasional pairing of converted isolates with "parent" virulent isolates seems advisable.

Field work with v-c has been less intensive because our knowledge in this area is just developing. Early work at Connecticut (Elliston and Jaynes 1977; Jaynes and Elliston 1978; 1980) utilized a single H strain to arrest canker development during a growing season. After three growing seasons and repeated treatments, 13 percent of the cankers (31:245) were arrested whereas none of the untreated control cankers was arrested. No attempt to match v-c groups of virulent and H isolates was made. Similarly, a second experiment in 1977 utilized eight H isolates from six v-c groups that were unselected for compatibility with virulent isolates. After two growing seasons, canker size and tree mortality were less in H isolate-wounded treatments than in nontreated, nonwounded treatments.

In the southern Appalachians, inoculated American chestnut trees have survived better following treatment with compatible H isolates or with a random selection of 28 H isolates than did check trees that were wounded and treated with agar (Kuhlman this proceedings). In the experiment started in 1978, one virulent-H combination was an erroneous choice that was not compatible and all the trees died. This emphasizes the need for ensuring compatibility for successful treatment. Since trees in check treatments died in 15 months, the need for rapid deployment of the H isolates to control virulent isolates is apparent. After several growing seasons, some live treated trees have signs of *E. parasitica* and symptoms of renewed infections. This renewed activity appears to be due to secondary infections by virulent isolates from other v-c groups. Whether inoculations with H isolates from several v-c groups can provide long-term protection against secondary infections remains to be determined.

Vegetative incompatibility is one impediment to control of chestnut blight with H in the eastern United States. Previous work has indicated that conversion of virulent isolates occurs 20 to 50 percent of the time between any two virulent and H isolates (Anagnostakis 1979; Grente and Berthelay-Sauret 1978; Kuhlman this proceedings; Puhalla and Anagnostakis 1971). Using the conservative 20 percent conversion, we calculated 14 randomly selected H isolates were needed to ensure 95 percent probability of conversion of any virulent isolate. In field studies, 28 H isolates have reduced tree death and girdling by virulent isolates. Long-term protection requires the survival of 14 H isolates according to this estimate. However, this estimate assumed each H isolate was independent of other isolates (i.e., each had a 20 percent possibility of converting any virulent isolate). Anagnostakis and Waggoner (1981) have indicated conversion is dependent on the number of similar v-c alleles in the two isolates. Thus two H isolates from v-c groups with only a single allele different are more likely to respond in the same manner to a virulent isolate than are two H isolates from v-c groups differing by five to seven alleles. Selection of H isolates from a broad spectrum of the 128 v-c groups might provide a 40 percent conversion rate and reduce the number of H isolates needed for control to six. Increasing our knowledge of the role of v-c may enhance the prospects for biological control of chestnut blight with cytoplasmic hypovirulence.

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CHESTNUT BLIGHT--PRIORITIES FOR RESEARCH

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ABSTRACT.--*This paper is an attempt to sharpen the original objectives and assign priorities for American chestnut research programs. A group discussion session was used to try to identify gaps in existing knowledge inhibiting American chestnut research progress, identify areas where research progress would indicate a high probability of success and where future research efforts should be directed. During the time frame of the meeting, it was not possible to obtain a specific concensus of research priorities. Solutions in one research area are required to solve problems in others. Priorities ultimately will be determined by the review process given to grant funded proposals.*

Introduction

Periodic assessment of progress toward objectives coupled with critical reevaluation of the original goals are necessary elements in all research programs. Often these steps lead to a sharpening or refocusing of the original research objectives--sometimes to a reordering of priorities.

The final discussion session of this meeting was devoted to such an examination. Its purpose was to identify (1) gaps in knowledge that are blocking research progress, (2) areas where research progress indicates a high probability of success with added effort, and (3) where future research efforts should be focused.

A schematic chronology of chestnut blight (Figure 1) was used to focus discussions on critical areas of the disease cycle that either influence or are influenced by the phenonemon of hypovirulence.

The original objective of the current chestnut blight research program was to shift, by means of hypovirulence, the outcome of infection from death to survival, that is, to increase the relative numbers of trees in categories (6), (8), and (10), as shown in Figure 1.

The following section presents the items discussed under each topic (numbers correspond to Figure 1), pertinent points that emerged in the discussions, and a statement of the topic's relative priority in future chestnut blight research on hypovirulence.

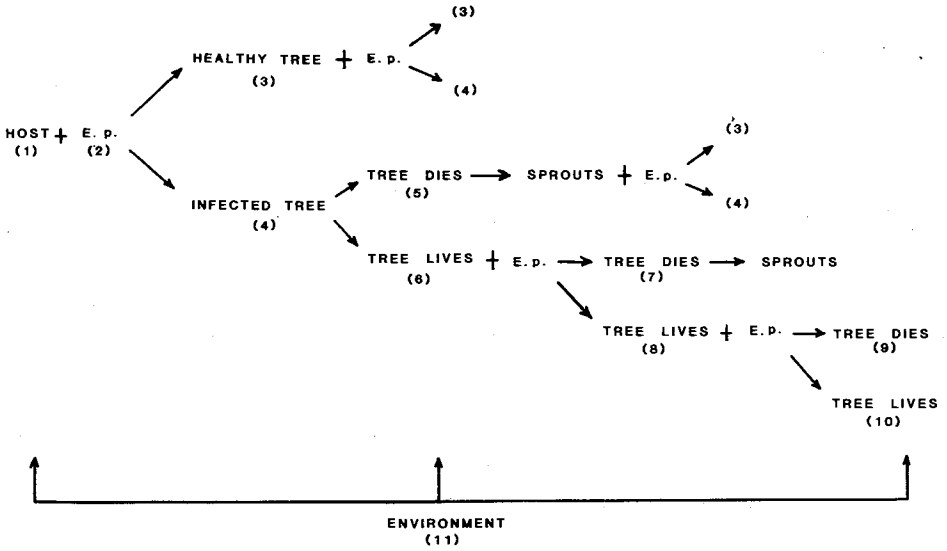


Figure 1. This scheme indicates that healthy host trees (1) exposed to the pathogen *Endothia parasitica* (E. p.) (2) either do not become infected (3) or do become infected (4). Infected trees develop cankers that either kill them (5) or do not kill them (6). The disease may repeat on sprouts from stumps of killed trees. Cankered trees that survive (6), either because they are genetically resistant or because the fungus is less virulent, are exposed repeatedly to reinfection, and either succumb (7), (9), and so on or survive (8), (10), and so on. Influencing all of these agents and their interactions are factors of the physical environment (11).

Topic (1) Factors that influence the relative susceptibility of host trees

a. Host genetics
b. Tree status (size, age, origin)
c. Ecological forest relations (density, distribution patterns, geography)
d. Non-American chestnut hosts (oaks, Chinese chestnut)

a. Evidently American chestnut is not resistant to virulent strains of *E. parasitica*.

- Because past efforts to find trees resistant to normal strains of *E. parasitica* have been unsuccessful, a major effort to locate trees that are resistant to hypovirulent (HV) strains probably is not warranted. However, researchers comparing host responses to HV isolates should distinguish between the effects of fungi debilitated to varying degrees, and effects attributable to different host genotypes.

Previous efforts in tree genetics focused on either discovering resistant American chestnut trees or incorporating resistance factors from other species into American chestnut genomes. With HV strains, the focus is shifted to discovering diseased trees with cankers that are superficial and persistent and that produce viable propagules of HV strains.

- With this shift in focus, host genotype may become more important. Researchers should recognize that it may now be possible to select desirable trees from within genomes previously considered undesirable.

b. The influences of such tree variables as size, age, and origin (sprout versus seedling) on relative susceptibility were alluded to in several papers presented at this meeting.

- The possible influence of such variables must be recognized and well documented because differences in host/pathogen interactions involving HV may reflect more critically, the differences in tree condition.

c. The numbers of chestnut trees per unit area, their distributions relative to each other (degree of aggregation or isolation--of individuals or stands), and the geographic locations (growing within or outside of the natural range) are variables that seem to influence the probability of infection.

- Dense stands or local aggregations of chestnut were previously considered undesirable because they favored spread, development, and persistence of the virulent pathogen. Such relationships may need to be reevaluated in light of HV. It may become necessary to manipulate these variables to ensure the spread and development of less competitive HV strains.

American chestnuts are growing in Michigan beyond the natural range of the species. In many former groves, trees were severely blighted--many were killed. But, in Michigan, in contrast to forests in Appalachia, some large trees and many of the sprouts from root systems of killed trees did not succumb to infection. Cankers from such survivors have often yielded HV isolates.

- The situation in Michigan provides the only clear parallel in this country to that reported in Europe. It is a dramatic natural demonstration that biological control, presumably HV, can arrest the spread and development of chestnut blight in American chestnut. It is very important to understand what is happening in the Michigan "Laboratory" with respect to the source of HV, mechanisms of transmittance, and the patterns of spread and development of HV over time within and between trees and stands. It may comprise the model needed to clarify the ecological relationships affecting the use of HV.

d. The significance of non-chestnut hosts--especially scarlet and post oaks--in the disease cycle and especially in relationship to HV is not clear. It is not known if oak cankers can be converted to HV types--and if so, whether they will continue to persist and serve as sources of HV inoculum.

- The probable role of oak-inhabiting insects (especially ants) as carriers, the basal location of many oak cankers, their persistent non-lethal nature, and their abundance in certain areas warrant a continued research effort to clarify the significance of oak hosts to HV.

Conclusion: Intermediate priority

- Except for studies on non-chestnut hosts-- and on understanding the situation in Michigan (see Topic 2 as well)-- the above items that deal in general ways with host tree susceptibility are somewhat lower in priority than those that relate to the pathogen itself (Topic 2) or to specific aspects of the host-pathogen interaction (Topics 4, 6).

Topic (2) Factors that influence epidemiology of HV *Endothia parasitica*

A. Field Level

a. Inoculum density
b. Inoculum type (propagules)
c. Means of dissemination
d. Infection courts
e. Seasonal effects
f. Ecological niche (saprophyte - parasite)
g. Vegetative compatibility (v-c)

B. Molecular Level

h. Molecular biology of hypovirulence
i. ds-RNA
   -its identification and characterization
   -its form in host
   -its means of transmission
   -its origin

2A: a-g. The basics to understanding HV epidemiology are: knowledge of how much inoculum is required to initiate HV infections in nature; what propagules are responsible and how they differ from virulent types; how these propagules are disseminated over short and long distances, to what infection courts, and at what time of year; in what niche HV is perpetuated; and what inter-strain factors regulate conversion. Such information is also requisite to the design of control programs utilizing HV.

Discussions of the factors influencing epidemiology of HV revealed that much of this information is either lacking or not readily available--not only for HV, but also for normal strains as well.

- We need to learn what is known about the epidemiology of normal strains so valid comparisons can be made to HV strains.

- It would be valuable to screen the old literature for pertinent information that, when reinterpreted in view of today's situation, may yield important clues. Often, vital information obtained by astute observers is hidden in out-of-print or obscure literature.

Basic epidemiological questions are difficult to answer--and often entail studies that are both money and labor intensive.

- A new approach may be necessary. We may need to reinstate the practice of patient observation utilized by our predecessors. Patience is also the key to demonstrating the efficacy of HV in the field. Because of the normal lag effect that always occurs in the development of disease epidemics, it will require time before an HV "epidemic" can be recognized or reach the stage of measurement.

The development and spread of HV probably will occur everywhere eventually, but recognition of these phenomena may be very difficult in areas where endemic "background counts" of virulent normal strains are innundative.

- The dynamic situation in Michigan may provide a special opportunity to "sit and watch". Good observations often result from seizing situations--sometimes fleeting--that are unique in time and place. The stage of disease development and the unusual HV and v-c relationships in Michigan make epidemiological studies there of high priority.

2B: h-i. How important is it that we understand HV at the molecular level? Do we need to know the nature of ds-RNA, how it occurs in its fungal hosts, how it is moved from one fungus to another--from one location to another, where it comes from?

- The phenomenon of HV does exist--its effects have been demonstrated both naturally (isolated individual diseased surviving trees in Appalachia; reduced tree mortality in Michigan) and artificially (conversion and 'control' of individual inoculated cankers in many locations and lowered mortality rates following massive dispersal of inocula in Connecticut. And, evidence of spread of HV following its artificial introduction is just beginning to accumulate. All of this has occurred with very little understanding of HV at the molecular level. Yet, until the questions posed above are answered, future approaches to using HV will remain strictly trial and error.

Information at the molecular level may be acquired relatively rapid because of the recent advances in techniques in these fields.

- Many new techniques have yet to be applied to this problem (for example, gene recombinant studies to reduce or interfere with virulence). Molecular level information, once acquired, must be placed in the context of natural systems through concommitent studies in field epidemiology. Integrating information from research at all levels will help to ensure that control programs utilize the right materials, at the right time, at the right place, in the right way.

Conclusions: High priority

- Research on factors affecting the epidemiology of HV strains of *Endothia parasitica* is a high priority. It is important to "relearn" what is already known about the virulent strain and to compare that to results from new studies on HV isolates. An understanding of the molecular biology of HV is of great importance to the intelligent development of effective methods to manipulate or manage HV at field levels. A multi-level research thrust will produce more results faster than will research at only one level.

Topic (3) Factors that either contribute to the occurrence of or relate to disease-free trees (survivors).

a. Resistance mechanisms
b. Escape mechanisms
c. Methods to vegetatively propagate healthy survivors (also desirable trees in other categories: hybrids for testing, trees with "desirable" cankers, and so on.)

a-b. It is unlikely that resistance or escape mechanisms will figure prominantly in research programs on HV--especially where abundant virulent inocula exist.

- Researchers challenging trees with debilitated isolates should be alert, however, to the possible influence of host genetics (see Topic 1).

c. Research has now demonstrated that American chestnut can be propagated *in vitro*. Continued research is needed to refine and improve these techniques.

- Clonal "testers" are needed for critical evaluation of specific HV factors; American x European hybrids should be multiplied to assess their responses to HV in different areas; and, because some surviving trees in Michigan have better form and quality than expected, it may become important to propagate and test them under a broader range of conditions.

Conclusions: Low to intermediate priority

- Except for efforts on vegetative propagation--which have applicability elsewhere as well--research on this topic is a relatively low priority.

Topic (6, 8, 10, and so on) Factors responsible for the survival of cankered trees

a. Host-parasite interaction: bark cankers
b. Factors influencing host responses
c. Desirable host responses

a. When infection by *E. parasitica* is successful, the host-parasite interaction results in formation of bark cankers. Whether infected trees die (5) or survive (6, 8, 10,...) depends on their individual responses to infections by particular parasites.

- Presumably, most non-lethal cankers reflect host responses to HV strains of *E. parasitica*. Non-lethal cankers differ in appearance and consequence. Some are deep but limited laterally, some are superficial but extensive and persistent, some are combinations. Some result in little apparent adverse effect on tree form and growth-- some markedly affect the growth habit and shape of the tree.

b. Variations in canker appearances and effects probably reflect variations in such host responses as callus formation and necrophyllatic periderm establishment.

- Such variable host responses may result from differences in: strains of *E. parasitica* ("factors" of HV, degree of debilitation); timing of infection (seasons, tissue phenologies); tissues infected (age, infection courts); interval between infection and response (time before tree "recognizes" infection); and tree condition (energy status).

c. The host responses (type of cankers) incited by HV strains versus those incited by virulent strains are "the bottom line" of this research program.

- Desired are cankers that (1) are superficial (do not kill cambium and seriously maim trees), (2) produce viable spores carrying HV, that have sufficient diversity in v-c to effect conversion of virulent cankers in the region of concern, and (3) bear these spores for long periods of time (comprise persistent reservoirs).

A search for trees with "desirable" disease characteristics is quite a different approach from that usually taken in forest pathology, but one which has parallels in other biological control systems (take-all decline of wheat, gypsy moth control by parasites and diseases).

- The development and maintenance in the forest ecosystem, via HV, of mature chestnut trees, even though they are misshapen and of poor timber value, is a desirable first step. Such trees would be the means for genetic interchange and serve as a source of valuable wildlife mast.

Conclusions: High priority

- This area of research--the host response--is considered a high priority. It is very important to understand why a particular tree responds differently to infection by different HV isolates, or why different trees respond differently to infection by specific isolates.

Summary

- It was not possible to obtain a "finely tuned" concensus of research priorities. Factors influencing the trees, the pathogens and their interactions are so intimately associated that research on each area is important for success in the HV program. Solutions in one research area are required to solve problems in others.

Priorities ultimately will be determined by the internal and external reviews given to grant proposals. Sources of funds for grants (other than USDA, Forest Service) should be explored--especially for proposals that are well prepared and scientifically competitive.

ABSTRACTS

BIMONTHLY INOCULATIONS OF VIRULENT AND HYPOVIRULENT ISOLATES OF *ENDOTHIA PARASITICA*

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ABSTRACT.--One virulent and seven hypovirulent isolates of *Endothia parasitica* were inoculated at 2-month intervals for 1 year into healthy American chestnut trees, to determine whether date of initiation affects subsequent canker development. Each isolate was inoculated individually at one of six points on each of nine trees at each inoculation date, by insertion of fungal mycelium into a 0.5 cm bark wound. Initial inoculations were made in August 1979, with subsequent inoculations at 2-month intervals, through June 1980. Growth measurements (length and width) and estimation of sporulation were recorded every 2 months for each inoculation, from initiation through August 1981. Invasion of host tissue was significantly greater when cankers were initiated during the growing season (April, June, August, and October), as compared to the winter inoculations (December, February) where little or no growth occurred. Asexual sporulation was not observed until April 1980 on cankers initiated in August and October 1979, and perithecia were first observed on those cankers in August 1980. Cankers initiated in April and June 1980 generally began sporulation in August 1980 and produced perithecia by October. Bark samples were taken from all 432 inoculation sites to determine if the isolates used for canker initiation could still be recovered from the inoculation sites. Recovery rates ranged from 94 percent for the virulent isolate, to only 16 percent for the hypovirulent isolate EP-43. The remaining six hypovirulent isolates were recovered on an average of 73 percent. Reisolation from inoculations made during the 2 winter months was comparable to recovery from the other four inoculation dates, indicating that although the fungus is still viable, winter inoculations do not result in canker formation.

TREATMENT OF ENDOTHIA PARASITICA CANKERS USING 4 HYPOVIRULENT SLURRIES

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ABSTRACT.--Four different mixtures of hypovirulent strains and water agar checks were used to treat naturally occurring *Endothia parasitica* cankers on American chestnut. The hypovirulent mixtures were incorporated into agar and blended to make four slurries that were comprised of either a mixture of debilitated white strains (Grente's B-type); a mixture of slightly pigmented Italian strains of intermediate pathogenicity; a pigment but debilitated strain mixture (Grente's JR type); or, a general slurry including all strains and several North American hypovirulent isolates. New cankers were treated at 1 to 2 month intervals as they developed from May to November by punching 0.5 cm holes to the cambium at 1.0 cm intervals around the canker margin and introducing the slurry treatment. Each center was measured at the time of treatment and then during May and November of subsequent years. All treatments have slowed canker expansion when compared to the water agar checks. The general and B-type mixtures have slowed canker expansion the most and also contain the greatest number of debilitated strains. Many cankers that appear to be checked during the season of treatment commence growth in subsequent seasons. The month of canker treatment appears to have little effect on the canker's response to treatment. The number of new infections that arise on trees with treated cankers continues to increase and eventually results in tree mortality. The most encouraging result is that in certain plots up to 14 percent of the new infections contain hypovirulent inoculum. Further data management and statistical analyses are underway.

ANTS AS VECTORS OF *ENDOTHIA PARASITICA*

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ABSTRACT.--Various agar media, namely, chestnut bark, tannic acid (0.5, 1.0, and 2.0 percent), pentachloronitrobenzene (PCNB) (10 and 50 ppm), and potato dextrose agar were compared for selective isolation of *Endothia parasitica* with 0.5 percent tannic acid agar giving best results and utility. Ants collected from cankered and uninfected scarlet oak and Chinese chestnut trees under varying weather conditions were plated on the medium. To date, the fungus has been isolated from *Aphaenogaster lamellidens* in greatest frequency and inoculum yield following the rain. Only collections from infected scarlet oak have been positive so far. Presently, mode of transmission is being studied by caging ants infested with *E. parasitica* in fresh wounds on scarlet oak and Chinese chestnut. Screening of host-associated ant populations for vector quantification will be continued. By exposing them to hypovirulent-strain inoculum, attempts will be made to obtain conversion of virulent-strain cankers on Chinese chestnut by field caging experiments.

DETECTION OF RNA DEPENDENT-RNA POLYMERASE ACTIVITY IN CRUDE EXTRACTS OF VIRUS-LIKE PARTICLES IN *ENDOTHIA PARASITICA* STRAIN EP-43

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ABSTRACT.--The RNA dependent-RNA polymerase activity has been detected in crude virus-like particle extracts of 14-day-old shake cultures of EP-43 grown in glucose yeast extract agar. Extracts were made by homogenizing mycelium in 1M sodium acetate (pH 5.0) in a bead-beater. The homogenate was subjected to a low speed spin. The supernatant was made 0.3M NaCl and PEG 8000 was added at the rate of 10 g/1,000 ml. The precipitate was collected and subjected to one round of differential centrifugation (10,000x g for 30 minutes and 27,000x g for 90 minutes). The RNA polymerase assay mixture contained ATP, GTP, CTP, ^{3}H-UTP, magnesium ions, EDTA and crude extract, all buffered in TRIS-HCl to pH 7.9. Incorporation of ^{3}H-UMP into TCA-insoluble RnA was shown to be actinomycin D insensitive suggesting that polymerase activity was not DNA dependent. Similar experiments done with *Endothia parasitica* strain 671B also detected incorporation of label into RNA, but to a lesser extent.

THE ULTRASTRUCTURE OF *ENDOTHIA PARASITICA*: GENERAL FEATURES AND VISUALIZATION OF VIRUS-LIKE PARTICLES IN A HYPOVIRULENT ISOLATE

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ABSTRACT.--Transmission electron microscopy was done to cytoplasmically compare a virulent (V) and hypovirulent (H) isolate of *Endothia parasitica*. Hyphae of V isolate 16-15-1 (West Virginia) and H isolate EP-4 (France) were preserved by freeze substitution and embedded in an Epon-Araldite medium. In both isolates, mitochondria, nuclei, and many other cellular constituents closely resembled those described for freeze substituted *Fusarium acuminatum* hyphae. A spitzenkorper region was identifiable at the extreme hyphal tip, and was composed of a roughly circular congregation of microvesicles surrounded by larger apical vesicles. Membrane profiles were smooth and centers of presumed Golgi activity were relatively flat and fenestrated. The vacuole-lysosomal system was similar to that reported in freeze substituted hyphae of the basidiomycete, *Laetisaria arvalis*. In comparing the isolates, two major points of contrast were observed. First, there was a close association between smooth cisternae and mitochondria in the H fungus. Second, aggregations of spherical, isometric virus-like particles (VLP's) were seen associated with endoplasmic reticulum and free in the cytoplasm of the H isolate, but not the V isolate. It is possible that the VLP's are responsible for hypovirulence in the EP-4 isolate. Other H isolates will be screened for VLP's and compared to V isolates to determine whether any consistent ultrastructural differences exist.