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

J. S. Russin[1], L. Shain[1], G. L. Nordin[2]

[1]Department of Plant Pathology and [2]Department of Entomology
University of Kentucky
Lexington, KY 40546

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 biweekly.

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

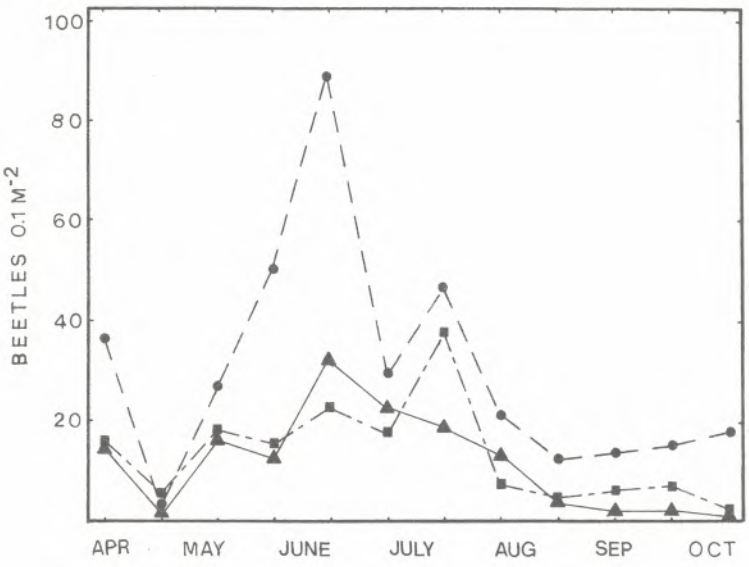

Figure 1. Total number of beetles trapped throughout the growing season from old blight cankers (● —— ●), young blight cankers (■— - —■), and healthy bark (▲——▲) of American chestnut.

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

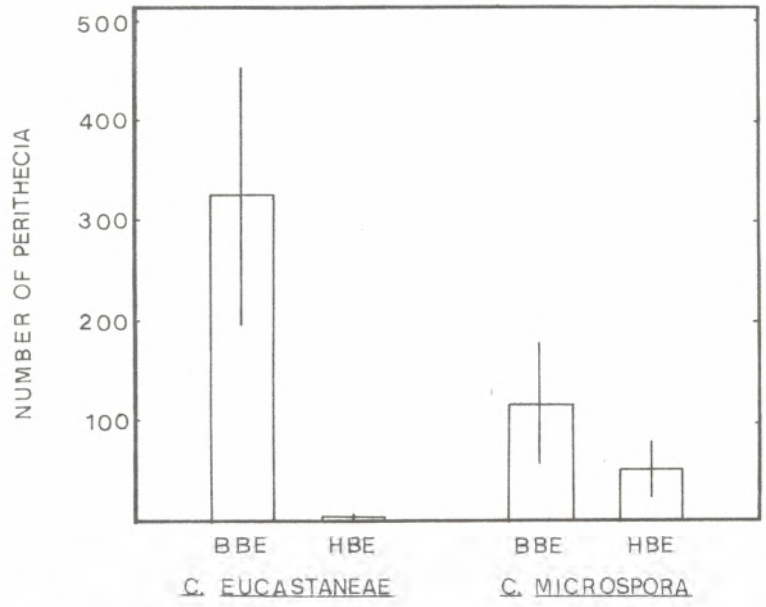

Figure 2. Perithecial production by *Ceratocystis eucastaneae* and *C. microspora* on media containing aqueous bark extracts of blighted (BBE) and healthy (HBE) bark of American chestnut.

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.

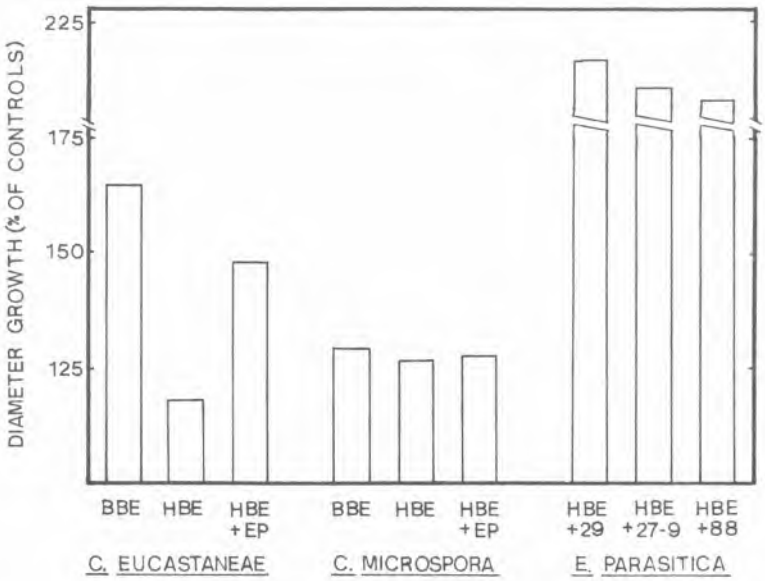

Figure 3. Diameter growth (percent of controls) of *Ceratocystis eucastaneae* and *C. microspora* on blighted bark media (BBE), healthy bark media (HBE), and healthy bark media which previously supported *Endothia parasitica* (HBE + EP). Values for *E. parasitica* represent diameter growth (percent of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

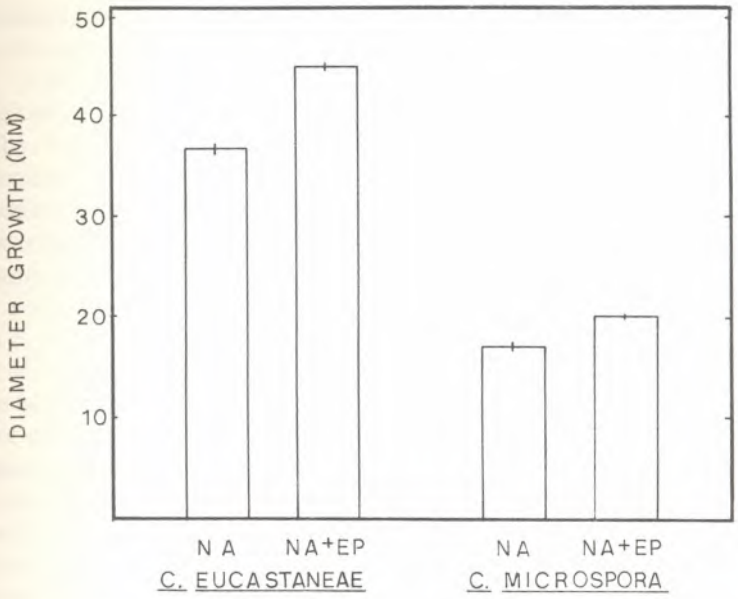

Figure 4. Diameter growth of *Ceratocystis eucastaneae* and *C. microspora* on Noble agar (NA) and Noble agar which had previously supported growth of *Endothia parasitica* (NA + EP).

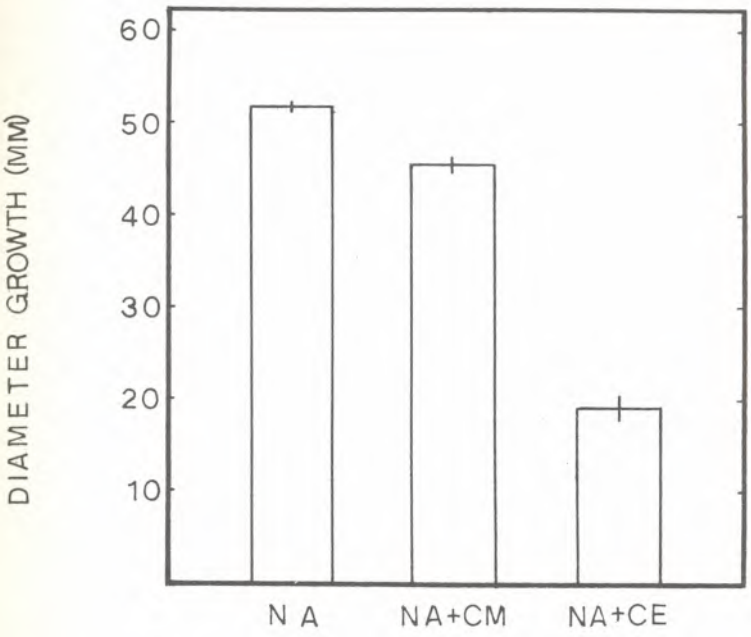

Figure 5. Diameter growth of *Endothia parasitica* on Noble agar (NA), and Noble agar which had previously supported growth of *Ceratocystis microspora* (NA + CM) or *C. eucastaneae* (NA + CE).

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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