Pathogenicity and Canker Control by Mixtures of Hypovirulent Strains of *Endothia parasitica* in American Chestnut

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ABSTRACT

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Pathogenicity of hypovirulent strains of *Endothia parasitica* on American chestnut stems decreased dramatically when two or more were mixed. Probably this effect is due to the exchange of genetic hypovirulence factors between strains possessing different factors; ie, superinfection, and fungal cell death that results from the interaction of strains that are vegetatively incompatible. Pathogenicities of normal and hypovirulent strains of *Endothia parasitica* were highly variable and strain dependent. Single hypovirulent strains often have been ineffective in controlling cankers, presumably because vegetative incompatibility hinders transfer of factors that confer hypovirulence. Mixtures of different hypovirulent mycelia effectively overcame vegetative incompatability and rapidly arrested canker development in both laboratory and field tests. However, mixtures tested to date may be too debilitated to survive and disseminate in the wild.

Biological control of the chestnut blight fungus, *Endothia parasitica* (Murr.) Anderson, on European chestnut, *Castanea sativa* Mill., has occurred naturally in Italy (10,11,13). French scientists discovered debilitated strains of the blight fungus in healing cankers in Italy, France, and Spain and designated them hypovirulent strains (10,11). Hypovirulence appears to be responsible for the decline of chestnut blight in Europe. Hypovirulent strains also have been found in North America (9). Collectively they display a wide range of abnormalities in cultural characteristics and degrees of pathogenicity (7).

Viruslike agents probably are responsible for hypovirulence in this fungus. All hypovirulent strains that have been tested contain double-stranded RNA (dsRNA) (5,6). When dsRNA is transmitted from a hypovirulent to a normal strain via hyphal anastomoses, the normal strain becomes hypovirulent (5,14). If the dsRNA is transmitted from a highly debilitated hypovirulent strain to a strain causing a normal blight canker, canker development can be arrested.

Vegetative incompatibility among strains of *E. parasitica* is common and to various degrees can hinder transmission of hypovirulence factors between individual strains (1–3). Presumably because of this incompatibility individual hypovirulent strains do not consistently control chestnut blight cankers (10,12).

In this study, mixtures of hypovirulent strains were laboratory tested for the ability to control cankers produced by representatives of 50 vegetative compatibility groups and field tested for ability to control cankers caused by strains whose compatibility groups were unknown. The effect that mixing hypovirulent strains has on their pathogenicity also was determined. Four experiments are reported: three on excised dormant chestnut stems and one, a 2-yr field test, on growing chestnut sprouts.

MATERIALS AND METHODS

Strains of *E. parasitica* were grown for 6–8 days in petri dishes on Difco potato dextrose agar amended with methionine and biotin at approximately 24 C (12,15). Agar containing mycelium of each strain was macerated in a Waring Blendor with enough sterile distilled water to produce a loose gel or slurry. Mixtures were made from equal parts of each component. Freshly harvested dormant trunk sections of American chestnut (*C. dentata* Borkh.) were used in Experiments I, II, and III. Cut chestnut stems were washed under running tap water and the cut ends and branch stubs sealed with embedding wax (7). Wounds 14 cm apart, 14–16 per stem, were made by removing 8-mm-diameter disks of bark to the depth of the sapwood with a cork borer. Wounds were filled with inoculum, the disks of bark were replaced, and covered with masking tape to retard drying. Each treatment was replicated three or four times, each replicate on a separate stem. Stems were placed vertically in a polyethylene-covered case containing moist paper towels to maintain high humidity, and incubated at 20 C. Canker growth, measured as length and width of necrotic tissue, was recorded at 1–2-wk intervals for 3–6 wk.

The effect that mixing strains has on their pathogenicities was determined in Experiments I and II. Also, the strains used in these two experiments were paired on agar by the technique of Anagnostakis (3) to determine if they were in the same or different vegetative compatibility (v-c) groups.

To test the ability of a mixture of hypovirulent strains to limit growth of normal strains representing many vegetative compatibility groups, a mixture of mycelial fragments of 10 hypovirulent strains was coinoculated with one representative each of v-c groups 1–50 (Experiment III) ([1]; and S. L. Anagnostakis, *personal communication*). The 10 hypovirulent strains included at least five v-c groups and the strains differed in pathogenicity (1,3,7). At each test site on a stem three slightly overlapping wounds were made in a vertical row with a cork borer. The normal strain was inoculated in the central wound and the mixture of hypovirulent strains in the upper and lower wounds. In the control treatment the normal strain was inoculated into the central wound and sterile agar was placed in the upper and lower wounds. Masking tape was applied and inoculated stems were incubated as described.

Long-term tests of hypovirulent and normal interactions in American chestnut trees were made in the field (Experiment IV). Virulent cankers were initiated on native chestnut sprouts and treated as previously described (8,12). Plots contained 24 sprout clumps. In May of 1977 each stem over 2.5 cm in diameter was inoculated at a height of 135 cm with an infected bark plug from natural cankers found within or near the plot; ie, normal, endemic, virulent strains were used, three different source cankers per plot. Laboratory testing confirmed that several v-c groups were represented in the sample. The technique was 99% effective in establishing cankers. Approximately 5 wk were allowed for the cankers to develop before hypovirulent treatments were applied.

Eight hypovirulent strains differing in pathogenicity were

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selected for the field treatments. These included French-derived American (15), native American, and Italian isolates, and represented six v-c groups. Treatments included: (treatment 1) an agar slurry containing mycelium of all eight strains was introduced into four circular wounds at 2 to 5 cm intervals around the canker margin; (treatment 2) inocula of the eight strains were applied individually, one in each of eight wounds, around the canker margin; (treatment 3) cankers treated with one hypovirulent strain, at least three cankers for each strain, and all eight strains were used in each plot; as for treatment 1, inoculum was placed in four wounds around each canker; and (treatment 4) plots that received no hypovirulent inoculum were identified as untreated controls. Each treatment, including the controls, was applied to three plots. The number of replicates (stems) per treatment ranged from 82 to 94. Measurements of canker length and width were taken in the fall of 1978, after two growing seasons, and canker areas were computed using the formula for the area of an ellipse.

RESULTS

Experiment I. Pathogenicity of single and mixed cultures of four hypovirulent strains of *E. parasitica* are given in Fig. 1. Data are presented as mean canker area bracketed by the standard error of the mean. The four strains tested, Italian strains 49 and 51 and American strains 88 and 92, were selected for relatively high pathogenicities on American chestnut stems (7). Each strain was in a different v-c group. Cankers produced by mixtures of two or more strains expanded less rapidly than did those produced by the individual component strains. Indeed, the mean growth of cankers caused by the mixtures always was less that that of the least pathogenic component except in one case, mixture of 51 plus 49, in which the growth was equal to the less pathogenic strain, 51. The effects of mixtures of three and four strains generally were less pathogenic and less variable among replications than those of mixtures of two strains.

Experiment II. Pathogenicity of single and mixed cultures of three normal strains and seven hypovirulent or suspected hypovirulent strains are given in Fig. 2. Data are presented as mean canker area bracketed by the standard error of the mean. In all but one case (bc) the growth of the mixtures was less than the mean growth of the component strains, regardless of whether the strains were hypovirulent or normal. Of the 10 strains used in experiment II only x and b were in the same v-c group, and growth of the xb mixture was essentially the same as the less pathogenic component. The mixtures of three (bcd) and four (fghi) hypovirulent strains were highly debilitated compared to the mixture (xyz) of three normal strains.

Experiment III. Effectiveness of a slurry of 10 hypovirulent strains in controlling growth of cankers caused by normal strains representing 48 different v-c groups is illustrated in Fig. 3. In this test, the pathogenicities of v-c group representatives differed considerably. Representatives of v-c groups 13 and 15 contained dsRNA. The mixture of hypovirulent strains reduced canker development in all cases. In only four cases did the standard errors of the treatment and control means overlap. The slurry(s) inoculum introduced alone grew very little.

Experiment IV. All treatments with hypovirulent strains (mixture of 8, 8 around, and individual) were significantly effective ($P = 0.05$) in reducing canker size: 59, 67, and 128 cm^2, respectively, compared to 442 cm^2 for the untreated cankers (Fig. 4). Canker area at the time of treatment with hypovirulent strains was approximately 19 cm^2. The use of eight strains, inoculated as a mixture or individually around a canker, was more effective in limiting canker size than was a single hypovirulent strain. Differences in canker area between treatments and control would have been even greater if cankers on killed trees had been included because more of the treated stems survived: 43–77% compared to 22% for the untreated (Fig. 4). Cankers on killed trees were excluded because they grew more rapidly after the stems had been girdled by cankers. Secondary blight infections were common in all plots (26 to 46% of the surviving stems) and were not related to the treatment.

DISCUSSION AND CONCLUSIONS

Mixtures of hypovirulent strains were consistently less pathogenic than the most pathogenic component of the mixture and usually less pathogenic than any of the strains in the mixture. A

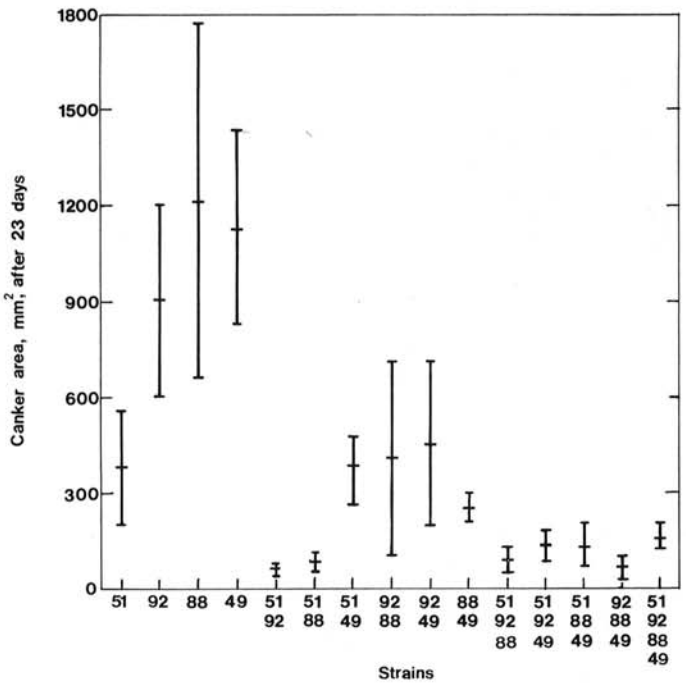

Fig. 1. Pathogenicity of single and mixed cultures of four hypovirulent strains of *Endothia parasitica* on excised stems of American chestnut. Bars indicate standard error.

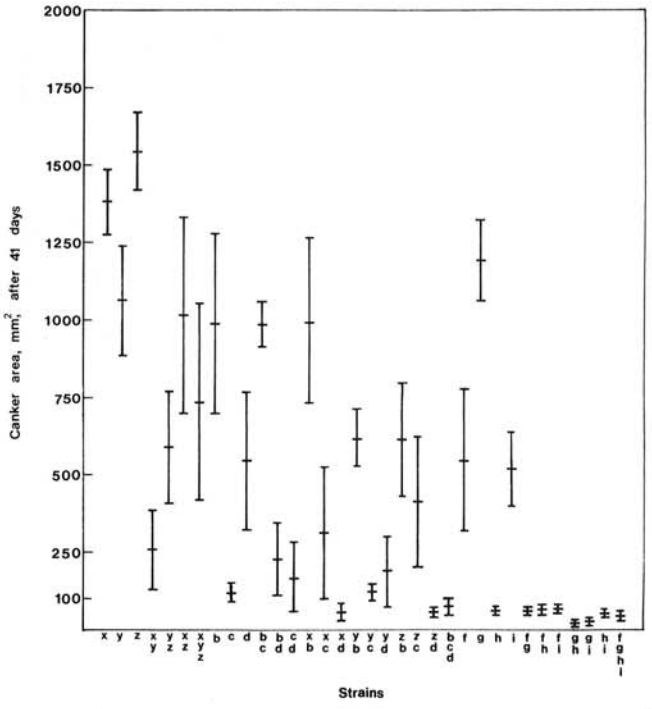

Fig. 2. Pathogenicity of single and mixed cultures of hypovirulent and normal strains of *Endothia parasitica* on excised stems of American chestnut (*E. parasitica* stock numbers—normal strains: x=6, y=37, z=149; hypovirulent: b=173, c=209, d and f=221, g=252, h=234, i=204). Bars indicate standard error.

mixture of three or more hypovirulent strains was almost always nonpathogenic, even if all of the components were relatively pathogenic.

A slurry of 10 hypovirulent strains applied to excised stems was universally effective in reducing growth of 48 normal strains classified in different v-c groups. In the field a combination of eight strains, whether applied as a slurry or individually to eight separate wounds around a canker, was highly effective in controlling cankers caused by normal endemic strains of *E. parasitica* on chestnut sprouts compared to inoculation with single hypovirulent strains. The problem of vegetative incompatibility, recognized as nontransfer of dsRNA and other traits from hypovirulent to normal strains, apparently was overcome by using slurries of hypovirulent strains.

At least two phenomena might be involved in the loss of pathogenicity by mixtures of two or more hypovirulent strains: death of hyphae caused by interaction of cells having different vegetative compatibilities, and the additive effect of different hypovirulence factors in the same mycelium. Two incompatible strains juxtaposed suffer cell death after hyphal anastomoses (10). Thus, thoroughly mixing mycelial fragments of two or more vegetatively incompatible strains probably causes debilitated vegetative growth. Even so, such mixtures control normal strains. Separation of the two effects could be achieved in part by using hypovirulent and normal strains of known v-c groups and comparing interactions within and between v-c groups.

Combining hypovirulent strains is effective in coping with the problem of vegetative incompatibility and, as shown, is a good method for controlling individual cankers. The minimum number of strains needed for transfer of hypovirulence to a range of v-c groups is not known, but it appears to be less than 10. Laboratory studies indicate that transfer of hypovirulence factors occurs between approximately 20% of incompatible strain pairs (4). Unfortunately, the sharp decrease in pathogenicity of mixtures of hypovirulent strains may reduce their capacity to establish themselves in the forest (7). If they do not grow well enough to

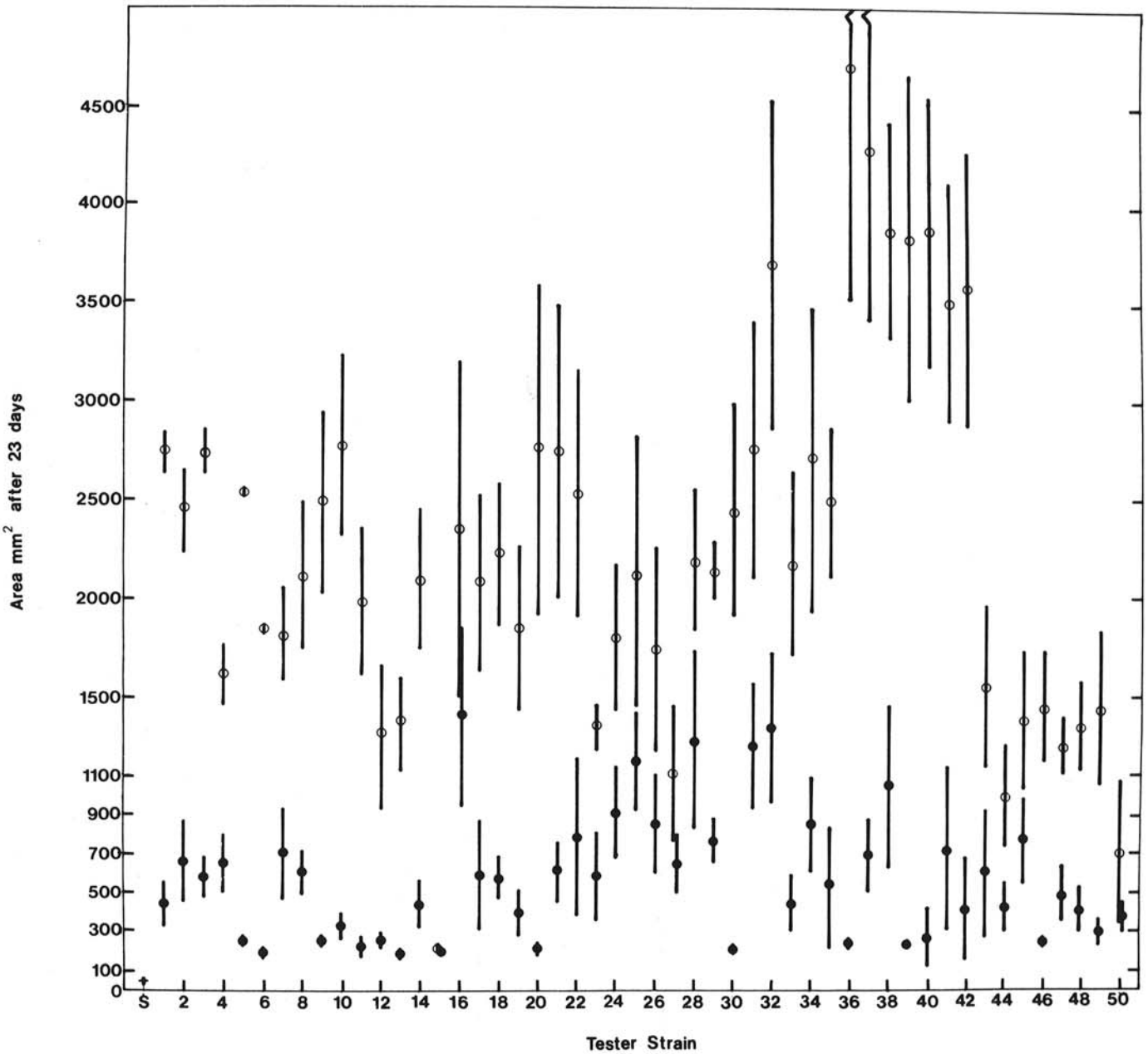

Fig. 3. Pathogenicity of vegetative compatibility tester strains 1–50 and effectiveness of a mixture of 10 hypovirulent strains of *Endothia parasitica* in controlling the growth of these strains on American chestnut stems. o = tester strain inoculated alone, • = tester strain coinoculated with mixture of 10 hypovirulent strains, S = mixture of 10 hypovirulent strains (Ep 3,4,14,43,53,60,90,94,166, and 167). Bars indicate standard error.

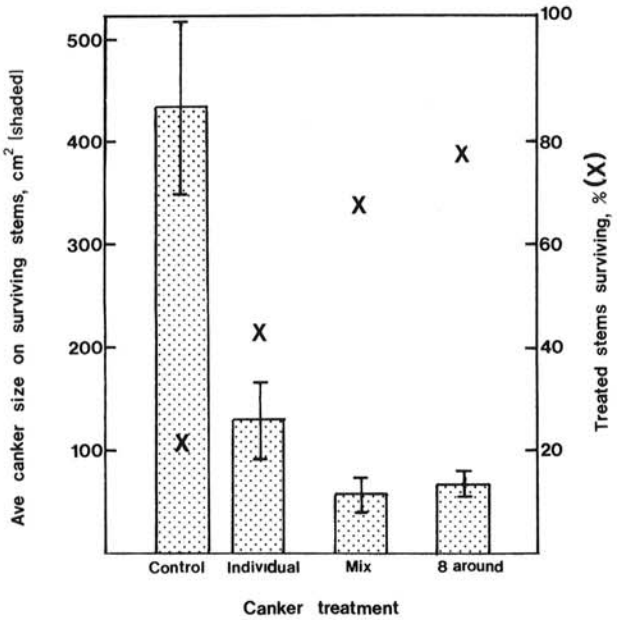

Fig. 4. Cankered area and percentage of American chestnut stems surviving two growing seasons after infection with normal strains of *Endothia parasitica* and treatment with hypovirulent strains. Bars indicate standard error.

sporulate and to maintain themselves vegetatively, then there is little chance that they will propagate and spread. Further study of the pathogenicity of single and mixed hypovirulent strains may lead to the goal of a hypovirulent inoculum that will control all normal virulent strains and still be sufficiently pathogenic to sustain itself in forest areas or orchards.

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