Localization of Infection in American Elms Resistant to Ceratocystis ulmi

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ABSTRACT

Branches of American elm trees previously identified as resistant or susceptible to *Ceratocystis ulmi,* and mainstems of 3-year-old ramets from these trees, were inoculated with bud-cell suspensions of the fungus. In branches of resistant trees, the interval from inoculation to appearance of foliar symptoms was longer, fewer shoots became symptomatic, and less extensive invasion of current-season shoots by *C. ulmi* occurred than in branches of susceptible trees. Resistant and susceptible trees differed in the proportion of shoots infected 4 days after inoculation; such differences increased during the growing season. When propagules of *C. ulmi* were flushed from segments of inoculated branches distal to points of inoculation, the number recovered from susceptible branches increased from 2 to 6 days after inoculation, while propagules from comparable resistant branches remained below detection levels. At 10 days after inoculation, 12 times as many propagules were flushed from susceptible, as from resistant, stem segments. Localization of natural infections in small branches and subsequent remission of symptoms were noted in four of the resistant trees. Differences in disease development and propagule recovery between ramets from resistant and susceptible trees were smaller than those between ortets. Young ramets of all clones were susceptible to *C. ulmi.*

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Beckman (3) proposed localization of infection as a general mechanism of resistance in vascular wilt diseases. He and others (4, 5) showed that restriction of internal spread of the parasite is important in resistance of several herbaceous plant species to wilt-inciting forms of *Fusarium oxysporum* Schlecht. In Dutch elm disease, localization of infection within small branches and remission of foliar symptoms are common in both susceptible and resistant trees (1, 7, 12, 21, 22). In susceptible trees, however, the localizing mechanism is ineffective during the seasonal period of maximum susceptibility, and systemic infections usually develop.

Slow suscept growth (13), rapid formation of tyloses after inoculation (10) and subnormal dimensions and contiguity of xylem vessel elements (8, 9, 11, 18) are associated with resistance in elms. Treatments that retard growth or induce tylosis formation enhance resistance (2, 6, 20).

Sinclair et al. (17) selected 17 American elms that repeatedly tolerated systemic Dutch elm disease. As a group, the selected trees grew slowly in comparison with nonselected neighbors, but growth rates varied widely among the selected trees (17, 23). In greenhouse tests, however, 1-year-old potted ramets from selected ortets were resistant only if inoculated after the seasonal peak of susceptibility (16). Therefore we reexamined the putative resistance of the selected ortets in comparison with nonselected elms. A preliminary report has appeared (19).

MATERIALS AND METHODS.—We studied events following inoculation of 2- to 4-year-old internodes (1 to 1.5 cm in diameter) on branches in the lower crowns of 54 elms 20-35 years old and on mainstems of young ramets from these trees. Sixteen of the trees had previously been selected as resistant to *C. ulmi* (17); 28 nonselected trees had grown up as sprouts after being cut or killed to ground level as a result of Dutch elm disease in the same selection program. Ten additional nonselected trees, presumed susceptible, had not previously been tested. One selected tree of *Ulmus laevis,* originally thought to be an atypical *U. americana,* was included with the American elms in several experiments. All trees were in plantations near Ithaca, New York. In six experiments, the number of trees ranged from 4 to 36. In three experiments, ramets from three each of selected and nonselected trees were used, and inoculations were made into their mainstems.

Inoculations and observation of foliar symptoms.—Branches chosen for inoculation bore at least five current-season shoots and were trimmed to leave a maximum of 10 shoots subtended by a twig-free distance of at least 60 cm to the proximal end of the branch. In most experiments, we chose branches that showed comparable rates of growth on selected and nonselected trees. Thus differences in symptoms or spread of the pathogen within inoculated branches would be independent of rates of growth of the inoculated branches.

The point of inoculation was about 30 cm proximal of current-season shoots. Three branches per treatment per tree were each inoculated with one drop of a bud-cell suspension of *C. ulmi* ($1.0 - 2.6 \times 10^3$ cells), prepared and administered as previously described (16). In some experiments, one or two drops of sterile water were also applied to the point of inoculation immediately following the inoculum drop. Noninoculated control branches, similarly prepared, received either sterile water or no treatment.

The number of days from inoculation to appearance of foliar symptoms (chlorosis, necrosis, abscission) was recorded. At intervals after inoculation, the proportion of shoots symptomatic on each branch was noted. A shoot was judged symptomatic if any leaf on it had symptoms. If

a branch remained nonsymptomatic, the duration of the experiment in days was recorded in place of the interval between inoculation and display of symptoms.

Internal distribution of C. ulmi.—The distribution of *C. ulmi* within inoculated branches was determined by incubating stem pieces on 1.5% water agar containing 200 mg cycloheximide per liter, and identifying the pathogen by its characteristic coremia (14). In one experiment, all stem tissue of the current growing season was cut into segments which were arranged serially in culture plates. In another experiment, cross-sections of stems about 5-mm thick were cut at 6-cm intervals from the proximal ends to the extremities of all current-season shoots and were plated in serial order. Information obtained for each cultured branch included the proportion of shoots and the proportion of stem sections or proportional length of shoot segments containing *C. ulmi.*

Proportional data were converted to arcsin $\sqrt{\text{percentage}}$ before statistical analyses. The noninoculated controls were used primarily to identify systemic infections and to monitor the frequency of natural inoculations made by the smaller European elm bark beetle, *Scolytus multistriatus* Marsh. The controls were not considered in statistical treatments, and are not reported with results.

Extraction of propagules of C. ulmi.—Propagules of *C. ulmi* were flushed from segments of inoculated branches or small mainstems by forcing sterile water through 10-cm stem lengths using either vacuum (8) or pressure from a nitrogen pressure bomb (PMS Instruments, Corvallis, Oregon). In the latter system, a reservoir of sterile water was placed in the pressure chamber. A debarked stem segment 10 cm long was inserted through a rubber stopper that served as a gasket. This was in turn fitted into the chamber cover which was locked in place such that the proximal end of the stem segment was in the water. The distal end of the segment, protruding through the chamber cover, was connected to a collection assembly which consisted of a 25-ml plastic graduated cylinder from which the base had been removed and replaced by a rubber fitting and flexible tube about 5 cm long. The end of the tube was fitted snugly over the protruding end of the stem segment and the assembly was clamped vertically. A pinch clamp on the tube allowed the assembly to be closed off after collection of the fluid.

With the collection assembly and a stem segment in place, the pressure in the chamber was raised to 3.4 atmospheres. Water was thus forced through the segment until 10 ml were collected. This quantity was stored at 2 C for up to 24 hours until diluted for plating of subsamples.

The collection assembly was rinsed once in 70% ethyl alcohol, and twice in sterile water, after each segment was flushed. Fresh sterile water was used in the reservoir each time.

To each sample was added 1 ml of 12.5% (v/v) Tween 20, plus sterile water to make the total volume 125 ml. The surfactant was used to disperse propagules. Ten plates per sample were each streaked with 0.1 ml of dilute suspension and colonies of *C. ulmi* were counted after 7 and 12 days of incubation at room temperature.

Localization of symptoms.—Branches of selected and nonselected trees and mainstems of nursery ramets were inoculated in early August with about 2×10^3 cells of *C. ulmi.* Three branches per tree or stems per clone received *C. ulmi;* one received sterile water. The branches and stems were harvested 12 days after inoculation, sealed in plastic bags, and stored at 2 C for up to 24 hours. A segment 6-10 cm long was cut from each branch or mainstem beginning 31 cm distal to the point of inoculation and was fixed in weak formalin-acetic acid-alcohol solution (15). Transverse sections 10- to 15- μm thick were cut, dehydrated, and permanently mounted on microslides. A 35-mm color transparency was made of one section from each segment. Discoloration in the xylem ring of the current season, as percent of area, was determined by projecting the image onto a grid and determining the percentage of grid squares showing

TABLE 1. Symptoms of Dutch elm disease and internal distribution of the pathogen in relation to shoot length in inoculated branches of white elms nonselected and selected for resistance to *Ceratocystis ulmi*

Experiment 1[a] Tree group	Mean shoot length at time of inoculation (cm)	Shoots infected 4 days after inoculation (%)	Days from inoculation to 1st foliar symptoms	Shoots symptomatic 46 days after inoculation (%)
Selected	11	20	32	32
Nonselected	19	47	16	66

Experiment 2[b]

Tree group	Length of longest shoot at time of inoculation (cm)	Shoots symptomatic 19 to 70 days after inoculation; avg. of 6 observations (%)	Shoots infected		Xylem sections infected 70 days after inoculation (%)
			4 days after inoculation (%)	70 days after inoculation (%)	
Selected	29	14 *	16	31	28 *
Nonselected	32	38	24	55	56

[a]Means of three branches on each of 10 trees per group. Each branch was inoculated in mid-June with one drop of a suspension containing about 10^3 bud cells of *C. ulmi.* All differences between selected and nonselected groups are significant, $P = 0.01$.

[b]Means of three branches on each of 16 selected and 20 nonselected trees. Each branch was inoculated in late June with one drop of a suspension containing 2.6×10^3 bud cells of *C. ulmi.* * Indicates difference significant, $P = 0.05$. Other differences were not significant.

discoloration within the image of the current-season xylem.

External symptoms and xylem discoloration were observed in three additional inoculated branches of each of five selected and five nonselected trees. Observations included the number of symptomatic branches on each tree and the linear extent of xylem discoloration above and below the point of inoculation in each branch 53 days after inoculation.

RESULTS.—*Disease severity in relation to apical growth rate.*—In the first of two experiments, branches of 10 selected trees, in comparison with 10 nonselected, showed slower rates of shoot growth, longer intervals between inoculation and appearance of foliar symptoms, fewer symptomatic shoots, and less invasion of current-season shoots by the pathogen (Table 1). Within the separate selected and nonselected groups, however, shoot length was not related to symptom expression or spread of the pathogen within inoculated branches ($r = 0$ to 0.11 in correlation analyses). Also, in the second experiment where branches of selected and nonselected trees were chosen for similarity of apical growth rate before inoculation, intergroup differences in disease expression were still evident (Table 1).

Distal spread of C. ulmi from points of inoculation.—The proportion of shoots infected (i.e., producing coremia when incubated) was closely correlated with both the proportion of total shoot length infected and the proportion of xylem sections infected ($r = 0.89$ to 0.95, $P = 0.01$). Therefore, only the data for proportion of shoots infected are shown (Fig. 1). As early as 4 days after inoculation, branches of selected trees had fewer infected current-season shoots than those of nonselected trees. When the trees were classified according to proportion of shoots infected 4 days after inoculation, seven selected trees (45%) and four nonselected trees (20%) were in the 0% and 1-9% classes, while the reverse trend (four and nine trees for selected and nonselected groups, respectively) occurred in the 25-100% infection class (Fig. 1-A).

These differences increased during the growing season (Fig. 1-B), primarily as a consequence of infection of many additional shoots on branches of nonselected trees. In the selected group, more than among nonselected trees, the proportion of shoots infected 4 days after inoculation was a determinant of the proportion of shoots infected near the end of the growing season, 70 days after inoculation ($r^2 = 0.51$ and 0.26, respectively). In both groups, many more shoots were infected than those showing foliar symptoms at the end of the season (Fig. 1-B, C).

Variation among trees, and among branches within trees, accounted for a large part of total variation in symptom expression and proportions of shoots infected. Thus, in Experiment 2, trees with 0-9% of shoots symptomatic 70 days after inoculation differed significantly ($P = 0.05$) from trees showing symptoms on 67-100% of shoots, but not from those showing intermediate responses. Seven of the 16 selected trees had 0-9% symptomatic shoots while two trees in that group displayed high susceptibility (91 and 93% of shoots symptomatic). In the nonselected group, two of the 20 trees showed 0-9% symptomatic shoots, while five trees showed symptoms on 67 to 100% of shoots. In six experiments, systemic infections developed in two selected ortets (12.5%) and nine (24%) nonselected ortets.

Recovery of propagules of C. ulmi.—In one experiment, propagules were extracted from 10-cm stem segments cut 21-31 cm above the point of inoculation 11 days after inoculation. One-of-10 selected trees and two-of-seven ramets from the selected trees yielded detectable numbers of propagules, whereas eight-of-10 and seven-of-nine of the nonselected ortets and ramets, respectively, yielded the pathogen. *C. ulmi* was recovered from 7% and

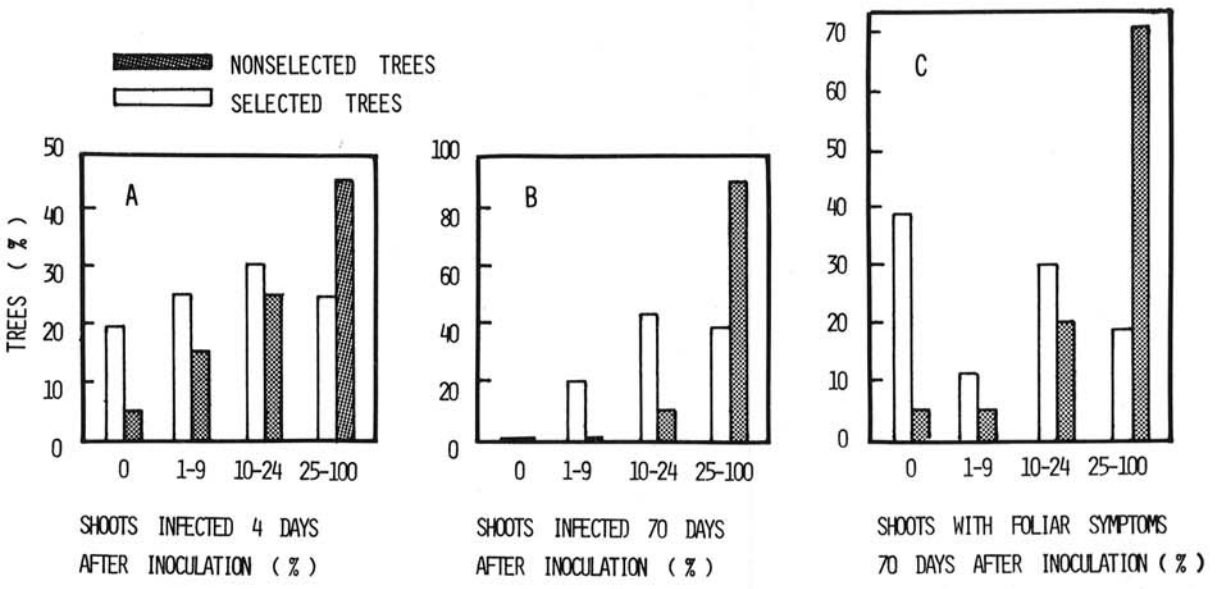

Fig. 1. Infection of current-season shoots and occurrence of foliar symptoms on inoculated branches of American elms nonselected or selected for resistance to *Ceratocystis ulmi*. Inoculation was made into current xylem on 2- to 4-year-old internodes 30 cm below the proximal shoot on each branch, using 2.6×10^3 cells of *C. ulmi*. Data are means from 16 selected and 20 nonselected trees.

TABLE 2. Propagules of *Ceratocystis ulmi* extracted from 10-cm segments of inoculated branches of ortets of *Ulmus americana* nonselected or selected for resistance to *C. ulmi*, and of mainstems of young ramets from selected and nonselected trees 11 days after inoculation.

Tree group	Propagules extracted[a]		
	From trees	From branches	Total colonies
Ortets			
Selected	1/10	2/29	5
Nonselected	8/10	18/30	133
Ramets			
Selected	2/7		3
Nonselected	7/9		97

[a]Fractions indicate recovery of *C. ulmi* in relation to total attempts.

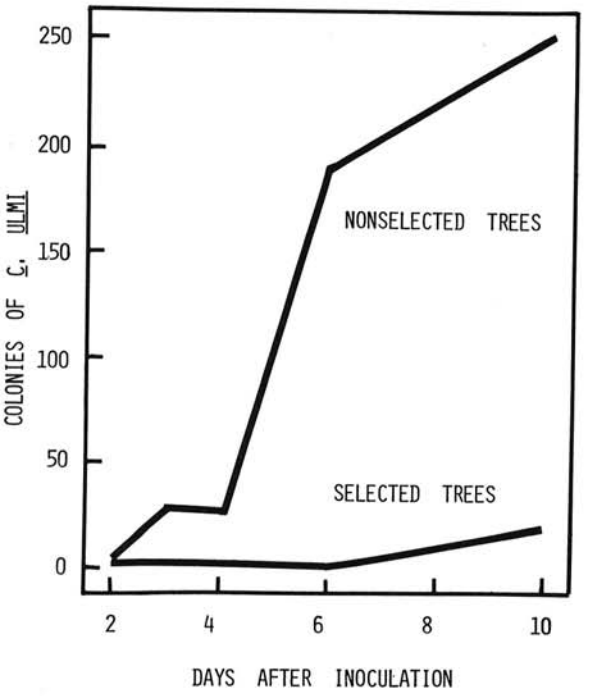

Fig. 2. Number of colonies of *Ceratocystis ulmi* from propagules flushed from segments of inoculated branches of white elms nonselected or selected for resistance to the pathogen. The data, pooled from two experiments, represent total propagules in 120 0.1-ml subsamples from diluted (1:12.5) 10-ml volumes of sterile water flushed through 10-cm segments cut 11-21 and 21-31 cm above points at which 10^3 bud cells of *C. ulmi* were inoculated into the xylem 2, 3, 4, 6, and 10 days before harvesting the branches. Three branches on each of two selected, and two nonselected, trees were sampled in each experiment.

60% of inoculated branches of selected and nonselected ortets, respectively (Table 2).

Similar trends occurred in two experiments that involved flushing propagules from 10-cm segments cut 1-11 and 21-31 cm above the point of inoculation 2-10 days after inoculation. Segments from inoculated branches of two nonselected trees yielded more propagules at each time of sampling than comparable segments from two selected trees. The number of propagules from branches of the selected trees was below detection level at 2, 3, 4, and 6 days after inoculation, and was only about 8% as great as from nonselected trees at 10 days after inoculation (Fig. 2).

Localization of symptoms,—In one of the experiments on propagule recovery, the proportion of cross-sectional area of xylem discolored in the current-season ring 12 days after inoculation was determined in one section per branch, about 35 cm above the point of inoculation. Five each of selected and nonselected trees were compared. One inoculated branch on each of two selected trees showed xylem discoloration which, however, encompassed less than 1.0% of the total cross-sectional area of the current season xylem in each case. Eleven branches on four nonselected trees showed discoloration of 5 to 14% of the cross-sectional area of the current season xylem. Six of nine ramets from three nonselected trees had 1-6% of the cross-sectional area of current season xylem discolored, whereas in two of seven ramets (three clones) from selected trees, discoloration was less than 1%. No discoloration was present at the point sampled in the other branches and mainstems. Xylem discoloration appeared as continuous arcs in sections from nonselected trees, and as isolated spots involving only a few vessels in sections from selected trees.

The proportion of shoots which were symptomatic, and the linear extent of xylem discoloration above and below the point of inoculation, were related to the amount of discoloration in the transverse sections 53 days after inoculation. One of 15 (7%) inoculated branches on selected trees, and nine of 15 (60%) branches on nonselected trees, showed foliar symptoms. Discoloration of xylem was seen an average of 15 cm below, and 58 cm above, the point of inoculation in selected branches; compared to 18 cm below, and 75 cm above, the point of inoculation in nonselected branches. The branches were 105 to 155 cm long, with the point of inoculation about 30 cm from the proximal end. The fungus became systemically distributed; i.e., caused xylem discoloration throughout the branch and beyond the branch at the proximal end, in seven nonselected and two selected branches.

DISCUSSION.—These studies corroborated the earlier report (17) of resistance in the selected trees. Localization of infection as the general mechanism of resistance is indicated by four lines of evidence: (i) failure to flush detectable numbers of propagules of *C. ulmi* from branches of resistant trees 2 to 6 days after inoculation, (ii) the relatively small proportion of shoots that became infected or developed symptoms on resistant trees; (iii) the substantial determination of final internal distribution of *C. ulmi* during the first 4 days after inoculation of resistant branches, but not susceptible branches; (iv) the limited staining of xylem in cross sections of inoculated branches of resistant trees, compared with more general xylem browning in susceptible branches. All of these results are compatible with the localization model proposed by Beckman (3).

We reported earlier that the xylem vessels in branches of resistant American elms were of smaller mean diameter than those in branches of susceptible trees (18). This difference, consistent with reports for resistant and susceptible elms of other species (9, 11), should enhance

the effectiveness of a localization mechanism since the time required for blockage of vessels would presumably vary inversely with vessel diameter.

During these studies, natural branch infections originating at feeding wounds made by *S. multistriatus* were found in four selected trees. In two trees, no foliar symptoms developed; the infections were discovered when noninoculated control branches were examined and cultured. In three other cases on two trees, foliar symptoms developed on branches up to 2 cm in diameter, but did not spread to larger branches. The symptoms persisted for only one growing season in each case. This remission of symptoms of natural infection was expected because remission of symptoms of systemic infections had previously occurred in the same trees (17).

The difference between selected and nonselected trees in recovery of propagules could be due either to rapid trapping of inoculum and localization of infection, or to less-rapid multiplication of the pathogen in the selected trees. The dilution scheme was such that growth of any colonies on the streaked plates could be taken as presumptive evidence of extraction of more propagules than the number applied as inoculum. Thus, rapid multiplication of *C. ulmi* in branches of the nonselected trees was indicated. However, we favor the former explanation because, in a study not reported here, we found no difference in the rate at which *C. ulmi* grew through living excised branch segments from resistant and susceptible trees. Elgersma (8) reported similar experience: no difference between resistant and susceptible clones of *U. hollandica* with respect to growth of *C. ulmi* in xylem sap.

Two selected trees developed severe symptoms characteristic of Dutch elm disease in most American elms. Both of these were formerly slow-growing trees in which vigorous shoot growth had been induced by fertilization. Susceptibility in this case was no surprise; it underscored the need for repeated rigorous infection tests as elms are screened for putative resistance to *C. ulmi*.

In all experiments involving inoculation of small ramets from selected and nonselected trees, differences between the two groups were smaller than between ortets in similar or parallel experiments. The relative lack of resistance in small clonal stock from resistant ortets, both in these field studies and in earlier greenhouse trials (16), is an anomaly we can not explain. The ramets will be tested again after several years of growth to learn whether they become more resistant with increasing age and size.

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