# Viability of Ceratocystis ulmi in Young Seedlings of American Elm and the Effects of Extracts from their Tissues on Conidial Germination

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ABSTRACT

Roots of 1- to 5-month-old American elm seedlings were inoculated with a spore suspension of *Ceratocystis ulmi*, and periodic isolations were made for 22 weeks. The fungus was recovered initially from 60 to 80% of the plants, but percentage recovery decreased in succeeding isolations. After 16 to 22 weeks, the fungus could not be recovered. Water extracts from leaves and stems of 1- to 5-month-old plants were fungistatic. Leaf extracts were fungicidal when their concentration was increased. No inhibition occurred with extracts from stems of plants 6 to 7 months old, stem and leaf extracts from plants 10 to 12 months old, or any root extracts. The inhibitor was dialyzable and heat stable at 98 C for 30 min. Survival of *C. ulmi* was also studied in rooted sprouts from inoculated 3- to 4-year-old American elms. The fungus was isolated from 95% of the sprouts immediately after sampling, from 62% 4 months later, and from 43% after 10 months. The more rapid loss in viability of the fungus appears to be associated with seedlings rather than with juvenile tissue in general. Unlike those from 1- to 5-month-old seedlings, extracts from sprout leaves and stems did not inhibit conidial germination. Foliar wilt and dieback did not occur in inoculated seedlings or sprouts. Phytopathology 60:31-35.

Difficulty in consistently producing Dutch elm disease symptoms in 1- to 3-year-old American elm, *Ulmus americana* L., seedlings has minimized their value in studies in greenhouses and growth chambers, and delayed the time for determining the results of breeding and selection programs (3, 4, 9). Juvenile resistance disappears in succeeding years, and these seedlings display the high level of susceptiblity characteristic of more mature trees.

Banfield (2) found that symptom development in mature trees was a function of vessel length, and that spores injected near the shoot apex, where vessels were short, produced no disease symptoms. Short vessels in young seedlings may similarly restrict spore movement. Smalley (7) associated susceptibility in young American elm seedlings with the period of branch elongation. He suggested that investigators might allow the susceptible period to pass before the start of their studies. Tchernoff (8) concluded that "youth resistance" was really insensitivity resulting from the way the plants were handled. Caroselli & Feldman (3) induced susceptibility in young elm seedlings by dark treatments prior to inoculation, but these results could not be duplicated by Heybroek (4). While the resistant reaction of elms to inoculation with *Ceratocystis ulmi* (Buis.) C. Moreau has been studied in 1- to 3-year-old seedlings, comparatively little work has been done on the susceptibility of seedlings less than 1 year old. Smalley (7) found that symptoms of American elm seedlings inoculated in their first growth year were confined to dwarfing, chlorosis, and incipient wilt.

This study was conducted to determine the distribution and survival of *C. ulmi* and the pattern of development of vascular and foliar symptoms of Dutch elm disease in American elms in their first growing season.

MATERIALS AND METHODS.—American elm seeds were germinated in vermiculite, fertilized weekly, and grown in the greenhouse under 500 ft-c and a 16-hr photoperiod.

Following germination, plants were inoculated at 4- to 5-week intervals for 5 months by placing the root system in a suspension of 500,000 spores/ml at 30 C for 18-20 hr. Isolations were made from stem sections and leaves of 25 plants immediately after inoculation to determine the number of infested plants. The rest were planted in vermiculite under polyethylene tents in shade until root development resumed, and then were returned to a 16-hr photoperiod. Fungus survival was determined by subsequent biweekly isolations from 25 plants. Twenty-five plants from each age group were observed for 1 year following inoculation to determine the appearance of foliar symptoms.

Extracts from stems, leaves, and roots of American elm seedlings, 1 to 12 months old, were tested for their effect on germination of *C. ulmi* conidia. Fresh tissue was diced, placed in distilled water under vacuum, and stored at 2 C for 19 hr. The liquid was decanted and reduced under vacuum at 34 C to 40 cc/100 g fresh wt, centrifuged at 3,200 rpm, and the supernatant was sterilized by filtering through a Gelman metrical membrane GA-6.

Extracts were bioassayed by applying 0.15 ml to a pad of six 1-cm squares of No. 1 Whatman filter paper. A drop of spore suspension (0.01 ml) was applied to a 1-cm square of nonmoistureproof cellophane (Brooks Paper Co., St. Louis, Mo.) on the filter paper pad. One hundred spores were counted on each of four cellophane squares to determine the percentage and type of germination for each treatment. The percentage of spore germination above or below that of the checks and the predominant types of germination (budding, unbranched germ tube, stunted germ tube or branched hyphae) were noted after 20-24 hr.

Survival of *C. ulmi* and the effect of extracts from leaves and stems on conidial germination were compared in rooted softwood cuttings and seedlings. Sprouts developed from adventitious buds following the inoculation of 3- to 4-year-old American elms. Isolations were made from 50 cuttings following their removal from inoculated trees, and the remaining sprouts were rooted in moist vermiculite. Survival of the fungus was determined by periodic isolations. Extracts from the

stem and leaf tissue of sprouts were prepared and tested as described for seedlings.

The fungicidal or fungistatic activity of leaf extracts from 2-month-old seedlings concentrated to 10 or 40 ml/100 g fresh wt was tested by the filter paper pad method described above. After 20-24 hr, germinated spores were counted on four cellophane squares, and these were then transferred to filter pads soaked in sterile distilled water. The remaining cellophane squares were transferred to pads with fresh extract. The following day the spores on water-soaked pads were counted. This process was repeated for 4 days to determine the effect of contact with the extract for 1-4 days. Extracts from 9-month-old elm leaves served as checks.

Survival of *C. ulmi* was determined in 3-month-old elm seedlings placed in the dark for 5 or 10 days prior to inoculation of roots. Following dark treatment and inoculation, all plants were returned to a 16-hr day. Isolations were made to determine the viability of the fungus.

The thermal stability of the inhibitor was determined by placing leaf extracts from 2-month-old plants in 100 μliter capillaries, sealing the ends, and treating them for 10 or 30 min in a water bath at 98 C. The samples were tested by the filter-paper-pad method for their effect on the germination of *C. ulmi* conidia. Unheated extracts were used as checks.

A 10-ml sample of inhibitory leaf extract was dialyzed by placing it in 1,500 ml of sterile distilled water at 6 C for 48 hr. The dialysate was reduced to 10 ml as described above, and the dialyzed material, the dialysate, and the nondialyzed extract were bioassayed for their effect on spore germination.

RESULTS.—*C. ulmi* was isolated from the stem, true leaves (Fig. 1), and cotyledonary leaves of elm seedlings immediately following root inoculation. Figure 2 gives the percentages of isolations of *C. ulmi* from elm seedlings in each age group, and the average of all of the groups. The most rapid decline in fungus viability occurred during the 2 weeks following inoculation. Viability subsequently decreased until the organism was no longer recovered after 16 weeks. There was no significant correlation between seedling age and the rate of loss of fungus viability. None of the plants from which isolations were made, or those held for observation for 1 year, showed extensive chlorosis, necrosis, or branch dieback. Vascular discoloration occurred sporadically in the stems of most inoculated plants, being more prevalent in those older than 12 weeks. Attempts to correlate stunting with the presence of the fungus, as reported by Smalley (7), were not successful because of the great variability in height within the inoculated and noninoculated groups.

Table 1 shows the effects of leaf, stem, and root extracts from elm seedlings 1 to 12 months old on spore germination. Spore germination was strongly inhibited by extracts from the leaves of plants 1 to 7 months old, but inhibition declined sharply in older plants, and disappeared from 10- to 12-month-old plants. Stem extracts were always less inhibitory than leaf extracts from the same plants. Their inhibitory effect declined most rapidly between the 3rd and 4th month; extracts

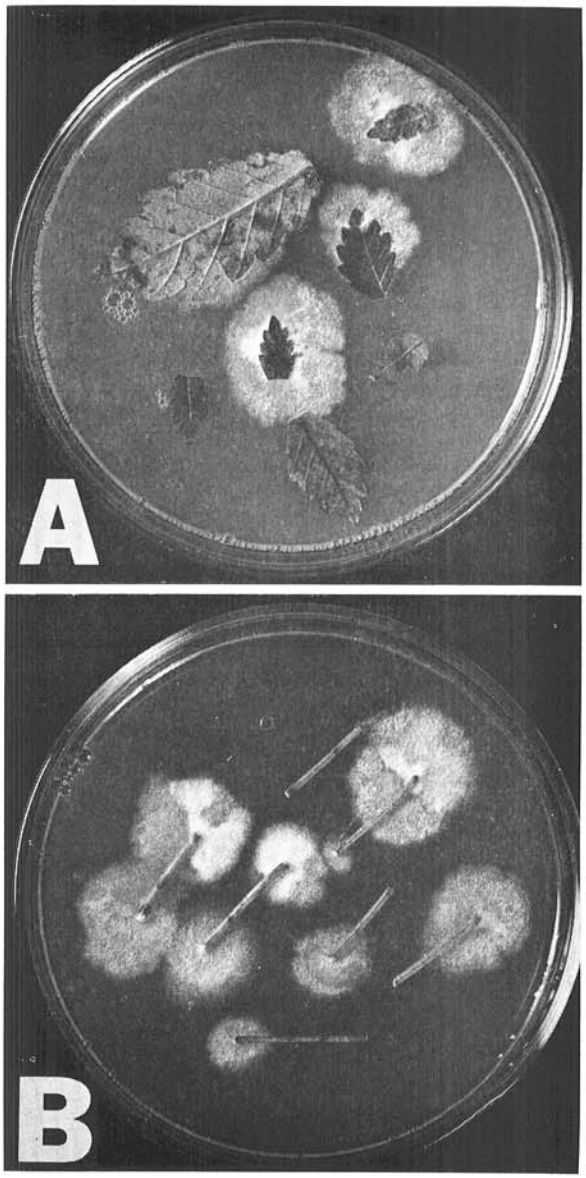


**Fig. 1.** *C. ulmi* growing from the leaves (A) and stems (B) of 3-month-old root-inoculated American elm seedlings.

from plants 6 months or older increased germination percentages above the checks. Root extracts from plants of all ages stimulated spore germination.

Extracts affected the type of conidial germ tube growth as well as the germination percentages. Inhibitory extracts caused conidia to produce stunted, swollen germ tubes, while those in contact with noninhibitory extracts produced normal germ tubes and hyphae. Water checks germinated by budding (Fig. 3).

Figure 4 shows the viability of *C. ulmi* in rooted softwood cuttings. The fungus was recovered from 95% of the cuttings after they were harvested from diseased trees. Recovery declined during the first 2 months to 43%, increased to 74% after 4 months, and declined to 43% after 10 months when isolations were discon-

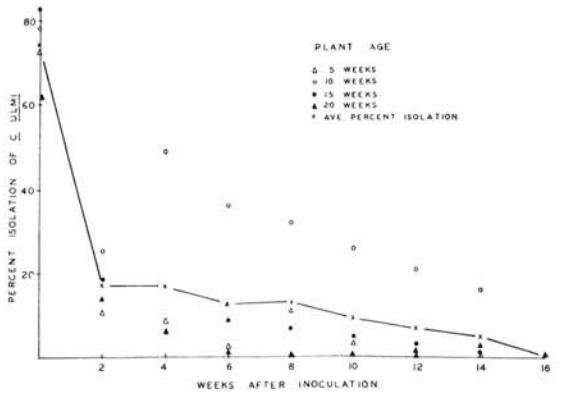


**Fig. 2.** Per cent isolation of *C. ulmi* from American elm seedlings. Seedlings of American elm, 5, 10, 15, and 20 weeks old were inoculated through their roots with a conidial suspension of *C. ulmi*. Periodic isolations indicated that the viability of the fungus decreased until it could not be recovered after 16 weeks.

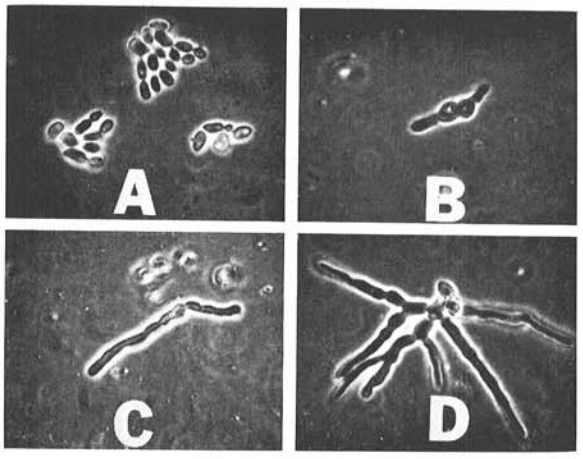


**Fig. 3.** Effect of various extracts from leaves, stems, and roots of seedlings of American elm on the growth of germinating *C. ulmi* conidia. **A)** Budding produced on water checks. **B)** Stunted germ tubes on inhibitory extracts. **C, D)** Normal germ tubes and branched hyphae on noninhibitory extracts.

tinued. The rooted cuttings did not show foliar symptoms, but did exhibit extensive discoloration in the wood adjacent to the pith. The leaf and stem extracts from rooted sprouts increased the per cent of conidial germination above the water checks and normal germ tubes or hyphae were produced (Table 1).

Leaf extracts from 2-month-old seedlings were fungistatic at 40 cc/100 g. Spore germination was greatly reduced below that in water checks at all exposure times, although a significant increase occurred after 4 days. Germination approached or exceeded that in checks when spores were placed on water-soaked pads for 24 hr following exposure to the extracts for 1-4 days. Spore germination was not reduced by the same concentration of leaf extracts from 9-month-old seedlings. Extracts from the younger seedlings concentrated to 10 cc/100 g were fungicidal after 1 day, as spore germination did not increase significantly after spores were transferred to water-soaked pads. Extracts at this increased concentration from the older seedlings delayed germination for 24 hr; but germination percentages approached or exceeded those of the checks when those spores were placed on water-soaked pads or if spores remained on the extract for longer exposure times (Table 2).

The survival of *C. ulmi* in 3-month-old seedlings was not affected by either a previous 5- or 10-day dark treatment. The fungus was isolated from over 95% of both treated and untreated plants after their roots were removed from the spore suspension and the percentage declined at about the same rate in both groups.

The activity of the inhibitory principle was not reduced by heat treatments of 98 C for 10 or 30 min or by storage up to 18 months at −15 C.

When inhibitory leaf extract was dialyzed against distilled water, the material in the cellophane membrane was devoid of activity and the activity in the dialysate was equal to nondialyzed extract.

Discussion.—Juvenile resistance connotes the absence of foliar symptoms in 1- to 3-year-old seedlings of susceptible elm species inoculated with *C. ulmi*. Vascular discoloration may be pronounced, and the fungus re-isolated throughout the plant. In other cases, lack of foliar and vascular symptoms result from limited spore movement and fungus growth (2). The existence of this phenomenon has been questioned since success in producing Dutch elm disease symptoms in juvenile seedlings, either in the field or greenhouse, has varied from one study to another (1, 3, 4, 5, 7, 8). Differences in results may be due to seedling variability, differences in seed sources, and cultural practices and environmental conditions under which the

Table 1. The effects of water extracts from leaf, stem, and root tissues of seedlings of American elms on the percent of conidial germination of *Ceratocystis ulmi*

| Type of tissue | % Germination above (+) or beow (−) controls Age of plants in months | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| Leaf | −48.6 | −75.1 | −85.6 | −82.5 | −83.4 | −84.6 | −91.7 | −12.8 | — | + 6.6 | — | + 4.2 |
| Stem | −22.0 | −33.0 | −20.0 | − 5.4 | − 2.2 | + 1.5 | + 1.0 | + 0.7 | — | +11.0 | — | + 1.1 |
| Root | + 5.2 | +11.8 | + 0.9 | + 1.3 | + 2.4 | + 6.0 | + 3.1 | + 3.6 | — | +11.3 | — | +11.0 |

The effect of extracts from sprouts of American elms previously inoculated with *Ceratocystis ulmi* on % conidial germination.

| | |
|---|---|
| Leaf | +9.5 |
| Stem | +3.0 |

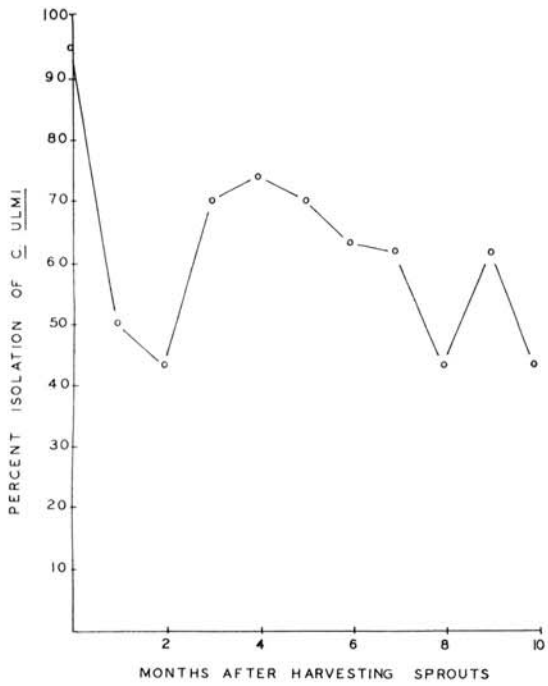


**Fig. 4.** Recovery of *C. ulmi* from rooted softwood cuttings harvested from diseased American elms. *C. ulmi* was isolated from a sample of sprouts that grew from adventitious buds on inoculated American elms 2 to 4 years old. The remaining cuttings were rooted and sampled monthly for 10 months to determine the viability of the fungus.

plants are grown. While plants of the same chronological age may be compared in different studies, variation in lighting, growth media, fertilization, or container size may produce physiological differences in age that affect disease development and symptom expression. Smalley (7) reported less severe symptoms in multibranched seedlings than in those grown from a single dominant bud.

With American elm seedlings in their first growing season, typical foliar symptoms were lacking, and vascular discoloration was common in plants inoculated after their 3rd or 4th month. Though *C. ulmi* was initially recovered from a high percentage of inoculated plants, viability decreased with time in plants under 5 months of age. This response may have resulted from several causes: (i) initially limited fungus distribution; (ii) a nutritionally deficient host substrate resulting in lack of fungus multiplication and production of disease-causing metabolites; or (iii) a fungal inhibitor in the younger seedlings that prevents disease development by eliminating the fungus.

*C. ulmi* was well distributed in leaves, stems, and cotyledonary leaves immediately following root inoculation. Though isolations gave no indications of inoculum levels in the plant tissues, fungus multiplication could normally be expected to increase to a level that would produce disease in a susceptible host. Schreiber & Stipes (6) found that as little as 1 ml of *C. ulmi* conidial suspension of $10^2$ spores/ml produced uniformly high Dutch elm disease symptoms in 4- and 8-year-old trees.

In vitro studies indicated that reduced fungus multiplication or viability in seedlings did not result from nutritional deficiencies, since conidial germination on a substrate of distilled water was generally above 85%. The per cent of conidial germination on a substrate of nutrient-rich plant extract would be expected to approach or exceed this. Higher germination percentage and branched hyphae were produced by extracts from older seedlings. Reduced spore germination and aborted germ tube development caused by leaf and stem extracts from younger seedlings indicate the presence of an inhibitor in these tissues. The inhibitory principle appears to be restricted to photosynthesizing tissue, and is in greater concentrations in leaves than in the stems. It may be produced in the former and translocated to the latter tissue. Root-tissue extracts from these plants increased spore germination above that of the checks. Since root extracts are noninhibitory, the principle is either not present or present at low concentrations in the roots. The inhibitory principle may retard spore germination or, if more concentrated, reduce fungus viability. Gradually reduced percentages of fungus recovery from root-inoculated seedlings indicated that the inhibitor may be fungicidal in vivo. The presence of an inhibitor in stems and leaves and reduced fungus viability were unique with young seedlings, and not characteristic of other juvenile tissue such as rooted softwood cuttings. Though Tchernoff (8) has questioned the truly juvenile nature of softwood cuttings because of certain anatomical similarities to adult trees, I based

TABLE 2. The effect of leaf extracts from 2- and 9-month-old American elms on germination of *C. ulmi* conidia

| Days in contact with extract | % germination above (+) or below (−) controls | | | | | |
|---|---|---|---|---|---|---|
| | 2-Month-old seedlings | | | | 9-Month-old seedlings | |
| | 40 cc/100 g[a] | After 24 hr on water-soaked pads[b] | 10 cc/100 g | After 24 hr on water-soaked pads | 10 cc/100 g | After 24 hr on water-soaked pads |
| 1 | −91.2 | −5.9 | −92.6 | −86.6 | −85.4 | 0 |
| 2 | −85.8 | +2.8 | −93.7 | −93.4 | +5.1 | 0 |
| 3 | −85.5 | +2.8 | −95.5 | −92.5 | −7.7 | 0 |
| 4 | −56.4 | +2.8 | −95.9 | −97.2 | +5.9 | 0 |

[a] Extracts from 2-month-old seedlings were concentrated to 40 or 10 cc/100 g from 9-month-old seedlings to 40 cc/100 g fresh wt of leaf tissue.

[b] Following exposure to the extracts, conidia were transferred to water-soaked pads for 24 hr and % conidial germination was again determined.

their juvenile classification on chronological age, their nonflowering state, and that of the parent trees from which they were harvested.

While rooted softwood cuttings did not develop foliar symptoms, vascular discoloration was prominent around the pith and the viability of *C. ulmi* remained high for 10 months. In addition, their tissue extracts did not inhibit conidial germination. The inhibitor, then, is not a common product of all juvenile tissue and may be unique in American elm seedlings. Failure of foliar symptoms to develop in the softwood cuttings may be attributed to successful walling-off of the fungus during the period of rapid growth. The causes of juvenile resistance in elms 1 to 3 years old may be varied, and may include limited fungus distribution, walling-off of the organism during periods of rapid growth, or the presence of fungistatic or fungicidal inhibitors.

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